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Attorney Docket No. 29473/11899A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with §1.6(a)(4).
Peter Mühlradt *et al.*	
Serial No. 10/748,033	
Filed: December 30, 2003	
For: Utilization of Lipopeptides or Lipoproteins in Wound Treatment and Infection Prophylaxis	July 1, 2009
Group Art Unit: 1654	Andrew M. Lawrence, Reg. No. 46,130
Examiner: Maury A. Audet	Attorney for Applicants
Confirmation No. 7597	

COMMUNICATION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This paper supplements the response filed June 30, 2009, in the above-referenced application.

The 131 declaration was not included in the response filed June 30, 2009. The applicants respectfully submit that the omission was inadvertent and that the same declaration was enclosed with the response filed September 8, 2008.

Respectfully submitted,

MARSHALL, GERSTEIN & BORUN LLP

July 1, 2009

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In the United States Patent Office

In Re Application of Peter F. Mühlradt and Ursula Deiters

Serial No: 10/748 033
Filed: Dec. 30, 2003
Title: Utilization of lipopeptides or lipoproteins in wound treatment and infection prophylaxis

Declaration

Professor Dr. Peter F. Mühlradt declares:

That he is a citizen of Germany, and that he is a biochemist and an expert in the field of woundhealing, and that he was an employee of Gesellschaft für Biotechnologische Forschung mbH (GBF), Mascheroder Weg 1, D 38124 Braunschweig, Germany, until January 31, 2002 to which the application identified above has been assigned.

Further he declares what follows.

The application identified above is a continuation application of US 09/716 779 filed May 10, 2001, which is continuation-in-part application of PCT/EP 99/03 436 filed May 19, 1999.

PCT/EP 99/03 436 characterizes the utilized lipopeptides and lipoproteins by their R-configuration which is in accordance with my understanding on the filing day as reflected by a publication by Takeuchi et al. with myself as co-author in J. Immunol., 164 (2000) 554-557 of Jan. 15, 2000.

Within the interval of from May 19, 1999 to Nov. 20, 2000 my attention was drawn to a publication by Metzger et al. in J. Med. Chem., 34 (1991) 1969-1974. On page 548 right column it is stated that product **3a** results from (S)-(-)-glycidol. Further, on page 547 right column it is stated that "In the following, **a** indicates the diastereomer with R configuration".

This statement leads to the conclusion that R-configurated MALP results from (S)-(-)-glycidol, but S-configurated MALP from (R)-(+)-glycidol. Since the active MALP as tested in the application as identified above was synthesized from (R)-(+)-glycidol, I thought that the correct configuration of the utilized lipopeptides and lipoproteins was the S-configuration. Consequently, US 09/716 779, the continuation-in-part application of PCT/EP 99/03 436 characterized the lipopeptides and lipoproteins by an S-configuration.

In order to get a clear impression of the stereochemistry involved in the work published by Metzger et al. and to confirm the S-configuration of the utilized lipopeptides and lipoproteins, I constructed stereochemical models with the result, that not the S-configuration, but the R-configuration was correct. As regards this understanding, attention can also be drawn to Deiters et al. in Experimental Dermatology, (2004) 1-10, who describe clearly a synthesis which starts from (R)-(+)-oxiran-2-methanol, i.e. (R)-(+)-glycidol and provides the active MALP2, and who confirm its R-configurationm; see page 2 "Material and methods". Further, Morr et al. in Eur. J. Immunol., 32 (2002) 3337-3347 published a stereochemical formula of the active MALP2 with its R-configuration; see Fig. 1 with its legend.

Professor Dr. Peter F. Mühradt further declares that all statements made herein of his own knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like, so made, are punishable by fine or emprisonment, or both under Section 1001 of Title 18 of the US code, and that such willful false statements may jeorpardize the validity of the application or any patent issuing thereon.

P. Mühlradt

Professor Dr. Peter F. Mühlradt Dated: 8/26/2008

1: J Immunol. 2000 Jan 15;164(2):554-7.

Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway.

Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, Mühlradt PF, Akira S.

Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Japan.

Mycoplasmas and their membranes are potent activators of macrophages, the active principle being lipoproteins and lipopeptides. Two stereoisomers of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 (MALP-2) differing in the configuration of the lipid moiety were synthesized and compared in their macrophage-activating potential, the R-MALP being >100 times more active than the S-MALP in stimulating the release of cytokines, chemokines, and NO. To assess the role of the Toll-like receptor (TLR) family in mycoplasmal lipopeptide signaling, the MALP-2-mediated responses were analyzed using macrophages from wild-type, TLR2-, TLR4-, and MyD88-deficient mice. TLR2- and MyD88-deficient cells showed severely impaired cytokine productions in response to R- and S-MALP. The MALP-induced activation of intracellular signaling molecules was fully dependent on both TLR2 and MyD88. There was a strong preference for the R-MALP in the recognition by its functional receptor, TLR2.

PMID: 10623793 [PubMed - indexed for MEDLINE]

J. Peptide Protein Res. 38, 1991, 545–554

Synthesis of N_α-Fmoc protected derivatives of *S*-(2,3-dihydroxypropyl)-cysteine and their application in peptide synthesis

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Received 14 December 1990, accepted for publication 15 July 1991

Acylated derivatives of *S*-(2,3-dihydroxypropyl)-cysteine (*S*-glycerylcysteine) form the *N*-terminus of structural and functional proteins of bacterial origin. Synthetic lipopeptides containing tripalmitoyl-*S*-glycerylcysteine are derived from bacterial lipoprotein and constitute potent immunoadjuvants activating both B-lymphocytes and macrophages. There is increasingly interest in conjugates consisting of tripalmitoyl-*S*-glycerylcysteine linked to appropriate viral and bacterial antigens, because of their capability of inducing antigen specific antibodies and T-helper and T-killer cell specific immune responses. A new convenient synthetic pathway for the preparation of these tripalmitoyl-*S*-glycerylcysteinyl peptides is described. The use of N_α-Fmoc-protected *S*-(2,3-dihydroxypropyl)-cysteine and its *O*,*O*′-bis acylated derivatives for the synthesis of triacyl-*S*-glycerylcysteinyl, *O*,*O*′-bis-acyl-*S*-glycerylcysteinyl and *S*-glycerylcysteinyl peptides of high diastereomeric purity by solid phase peptide synthesis or synthesis in solution is demonstrated.

Key words: Fmoc-*S*-glycerylcysteine; ion spray mass spectrometry; lipopeptides; MS/MS; peptide synthesis; *S*-glycerylcysteine, *S*-(2,3-dihydroxypropyl)-cysteine; synthetic immunoadjuvants

-(2,3-dihydroxypropyl)-L-cysteine (Dhc, '*S*-glycerylysteine') is an important amino acid of bacterial rigin. It is found in several proteins, especially structural proteins of Gram-negative bacteria (1, 2), such s the well-known murein lipoprotein from the outer embrane *Escherichia coli* (3) or the cytochrome ubunit in the photosynthetic reaction center from urple bacterium *Rhodopseudomonas viridis* (4).

In lipoprotein both hydroxy groups and the N_α-mino group of *S*-(2,3-dihydroxypropyl)-cysteine are cylated by fatty acids forming the *N*-terminal lipomino acid 'triacyl-*S*-glycerylcysteine'.

Synthetic lipopeptides containing *N*-terminally the ipoamino acid *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-propyl]-cysteine (Pam_2Cys-OH) (5) showed B-lymphocyte and macrophage stimulating properties comparable to native lipoprotein (5–8). Lipooligopeptides such as Pam_2Cys-Ser-Ser-Asn-Ala (5) and especially the water-soluble Pam_2Cys-Ser-$(Lys)_4$ (8) can replace other powerful but harmful adjuvants (9, 10); admixed to conventional vaccines the amount of the applied dose of vaccine may be considerably lowered (11). In addition, Pam_2Cys was successfully used as covalently linked adjuvant in fully synthetic, low-molecular mass vaccines (12, 13) and gained great importance for the *in vivo* priming of cytotoxic T-lymphocytes (14).

Pam_2Cys-peptides can be obtained by solid phase synthesis or by synthesis in solution using the synthetic lipoamino acid Pam_2Cys-OH in the last coupling step (5, 6, 8). In order to have more versatile building blocks for the preparation of *S*-glycerylcysteinyl peptides at hand, we elaborated two different synthetic approaches for the preparation of N_α-Fmoc-*S*-(2,3-dihydroxypropyl)-L-cysteine **1** (Fmoc-Dhc-OH) and of N_α-Fmoc-*S*-[2,3-bis(palmitoyloxy)propyl]-L-cysteine **5** (Fmoc-Dhc$(Pam)_2$-OH) in high diastereomeric

Abbreviations: Dhc = *S*-(2,3-dihydroxypropyl)-cysteine; DIC = *N*,*N*′-diisopropylcarbodiimide; Fmoc = fluorenylmethoxycarbonyl; HONB = *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide; IS-MS = ion spray mass spectrometry; Pam = palmitoyl; Pam_2Cys = *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)propyl]-[R]-cysteinyl; Ste = stearoyl; Lau = lauroyl; TDM = 4,4′-bis(dimethylamino)-diphenylmethane; Tfa = trifluoroacetic acid; TLC = thin-layer chromatography.

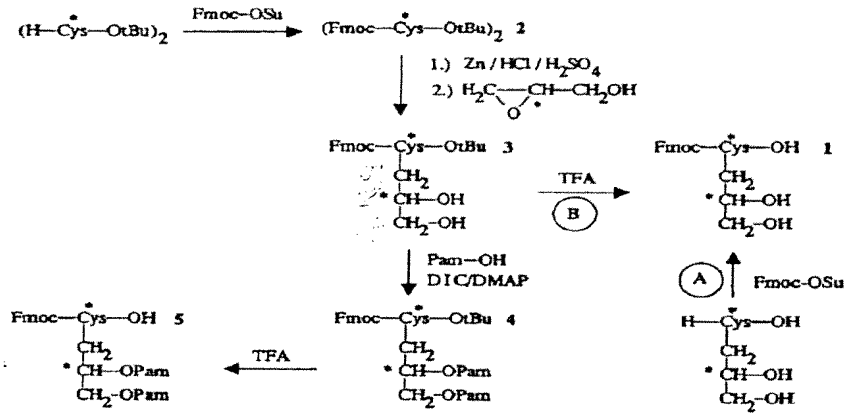


FIGURE 1
Synthesis of N_α-Fmoc-*S*-(2,3-dihydroxypropyl)-cysteine 1 and N_α-Fmoc-*S*-(2,3-bis(palmitoyloxy)-propyl)-cysteine 5.

purity (Fig. 1). These novel amino acids were used in synthesis in solution (Fig. 2) and in solid phase peptide synthesis (Fig. 3) to build up peptides containing the *S*-glycerylcysteinyl moiety.

EXPERIMENTAL PROCEDURES

Chemicals

All solvents used for synthesis and chromatography were of p.a. quality and purchased from Merck (Darmstadt, FRG). (*S*)-(-)-glycidol, (R)-(+)-glycidol, racemic glycidol, lauroyl chloride, stearoyl chloride, and palmitic acid were obtained from Aldrich Chemie (Steinheim, FRG). Fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide and H-Ser(tBu)-OtBu were purchased from Bachem (Bubendorf, Switzerland). Dimethylaminopyridine, *N*-ethylmorpholine and *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide were obtained from Fluka (Neu-Ulm, FRG). Sephadex LH-20 was obtained from Pharmacia (Freiburg, FRG). *S*-(2,3-dihydroxy-(2R,S)-propyl)-cysteine (20) and L-cystine bis-*tert.*-butyl ester were synthesized (5, 15).

Thin-layer chromatography

Silica gel plates 60 F_{254}, 5 × 10 cm (Merck, Darmstadt) and the following solvent systems (v/v) were used in solvent saturated glass chambers for determination of R_F values at room temperature. I, chloroform/methanol/water (65 : 25: 4); II, chloroform/methanol/acetic acid (90 : 10 : 1); III, ethyl acetate saturated with water; IV, chloroform/methanol (10 : 1); V, chloroform; VI, 1-butanol/acetic acid/water (2 : 1 : 1). For detection the plates were sprayed sequentially with ninhydrin reagent and chlorine/4,4'-bis(dimethyl-amino)-

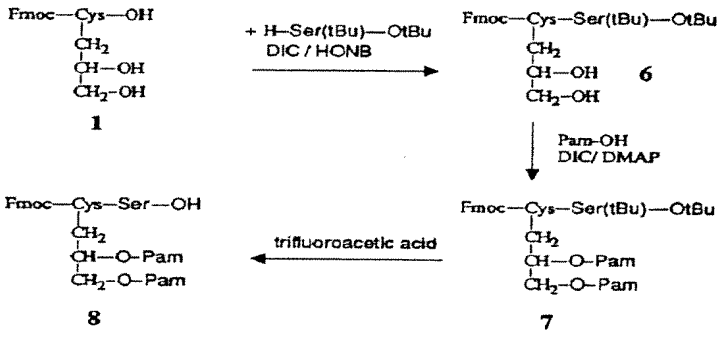


FIGURE 2
Synthesis of N_α-Fmoc-*S*-(2,3-bis(palmitoyloxy)propyl)-cysteinyl-serine 8.

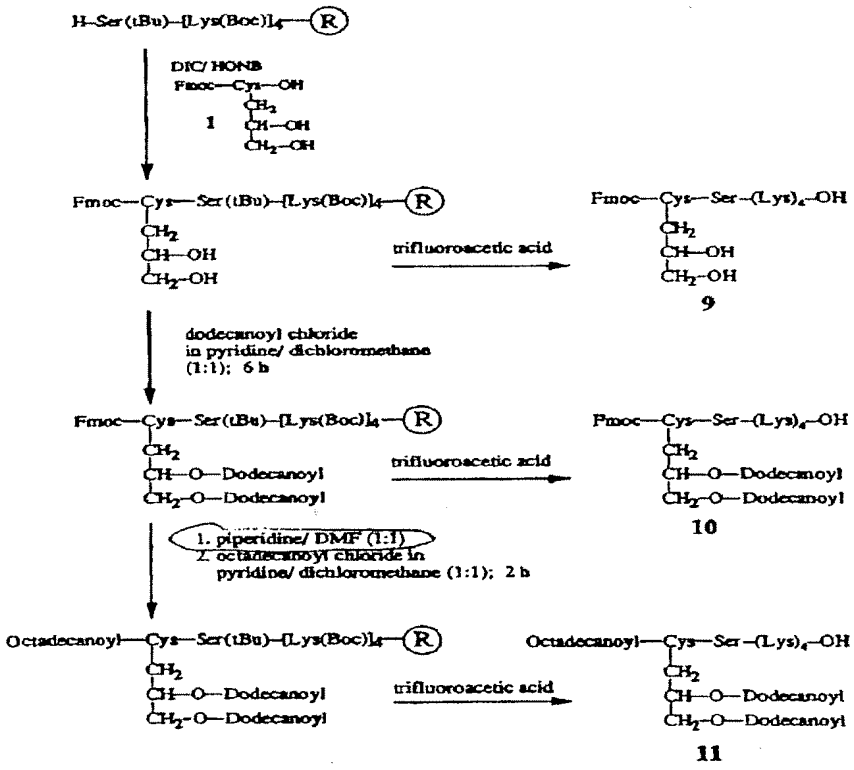


FIGURE 3
Synthesis of amphiphilic lipopeptides **9–11** by *O*- and *N*-acylation directly on the resin.

diphenylmethane (TDM reagent). Fmoc-containing peptides were additionally detected by UV (354 nm).

Analytical methods

The synthetic lipopeptides **8-11** were characterized by gas chromatography after total hydrolysis (6 N HCl, 110°, 18 h, in the presence of phenol), whereby the *S*-glycerylcysteinyl residue is partially decomposed (*5*). The obtained amino acids were automatically derivatized to the *N*-pentafluoropropionyl amino acid *n*-propyl esters. Their quantity as well as their optical purity was determined on a Chirasil-Val column (17) using enantiomer labeling (18). Optical rotations were measured in a 0.1 dm cell on a Perkin-Elmer 241 Spectropolarimeter. Field desorption mass spectra were recorded on a Varian MAT 711A (Varian, Bremen, FRG) at an ion source temperature of 50°. Ion spray mass spectra and ion spray tandem mass spectra were recorded on a Sciex API III triple-quadrupole mass spectrometer with 2400 Da mass range equipped with an ion spray ion source (Sciex, Toronto, Canada). The samples for ion spray mass spectrometry were dissolved in water/methanol (1:1), more lipophilic derivatives were dissolved in chloroform/methanol/10% aqueous formic acid (2:3:1; in some cases instead of formic acid 1% ammonium acetate was used). Final analyte concentrations were about 100 micromolar. The solution was introduced into the ion spray source at a constant flow rate between 1 and 5 μL/min with a microliter syringe using a medical infusion pump (Harvard Apparatus, USA). Total sample consumption was in the pmol range. Argon at a target gas thickness of about 1.0×10^{14} atoms cm^{-2} was used as collision gas for tandem mass spectrometry.

^{13}C NMR spectra were measured on WP80 (20.115 MHz for ^{13}C NMR) and WM 400 (100.16 MHz for ^{13}C NMR) spectrometers (Bruker-Physik, Karlsruhe, FRG).

In the following, **a** indicates the diastereomer with R configuration and **b** that with S configuration at C-2 of the *S*-glyceryl moiety; the mixture of the diastereomers is labeled **a/b**.

N_α-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxy-(2R/2S)-propyl]-[R]-cysteine (Fmoc-Dhc-OH), **1 a/b** *(Procedure A)*

1 a/b was prepared according to a general procedure for the preparation of Fmoc-amino acids, which has been described previously (19): *S*-(2,3-dihydroxy-(2R/2S)-propyl)-[R]-cysteine (20) (820 mg; 4.2 mmol) was dissolved in 9% sodium carbonate (10 mL). A solution of fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (1.18 g; 3.5 mmol) in dioxane (10 mL) was added. The mixture was stirred for 2 h, then diluted with water (120 mL), and extracted with ethyl acetate (3 × 25 mL). The aqueous phase was acidified to pH 2 with concentrated hydrochloric acid. The aqueous phase was extracted with ethyl acetate (6 × 25 mL). The extract was washed with saturated sodium chloride solution (3 × 20 mL), water (3 × 20 mL), dried over sodium sulfate, and evaporated to dryness. Recrystallization from chloroform or dichloromethane at −20° yielded a colorless powder. m.p. 22°. Yield: 1.38 g (94%). $C_{21}H_{23}NO_6S$ (417.5). IS–MS: $[M + H]^+$ 418.5. R_f(I) = 0.16; R_f(II) = 0.04.

^{13}C NMR ($CDCl_3$; 200 mg/mL; 256 scans; 20.115 MHz): δ(ppm) = 35.1 (*S*-glyceryl CH_2); 35.6 (Cys-C_β); 47.0 (Fmoc-C-9); 53.8 (Cys-C_α); 65.0 (*S*-glyceryl CH_2-OH); 67.4 (Fmoc-CH_2-O); 71.3 (*S*-glyceryl CH-OH); 120.0 (Fmoc-C-4,5); 125.2 (Fmoc-C-1,8); 127.1 (Fmoc-C-3,2,6,7); 127.8 (Fmoc-C-3,2,6,7); 141.2 (Fmoc-C-4a,4b); 143.8, 143.6 (Fmoc-C-8a,9a); 156.4 (Fmoc-CO); 173.3 (Cys-CO).

*N,N'-Bis(fluorenylmethoxycarbonyl)-[R]-cystine-bis-*tert.*-butyl ester ((Fmoc-Cys-OtBu)$_2$)*, **2**

L-Cystine bis-*tert.*-butyl ester (3.52 g; 10 mmol; refs. 5, 15, 21) and fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide ester (5.4 g; 16 mmol) were dissolved in tetrahydrofuran (10 mL). A solution of *N*-ethylmorpholine (2.55 g; 20 mmol) in tetrahydrofuran (5 mL) was added. After 3 h the mixture was evaporated to dryness. The residue was dissolved in ethyl acetate and washed three times with 5% $KHSO_4$ and water. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was recrystallized from dichloromethane/methanol (1 : 4; 300 mL) at −20°. The precipitate was washed twice with *tert.*-butanol/2-propanol (1 : 1) and dried over P_4O_{10}/KOH. Yield: 6.9 g (87%). R_f(I) = 0.89; R_f(II) = 0.81; R_f(IV) = 0.80; R_f(V) = 0.21. $C_{44}H_{48}N_2O_8S_2$ (797.0). IS–MS: $[M + H]^+$ 798. $[\alpha]_D^{20}$–6 (*c* 2, $CHCl_3$).

^{13}C NMR ($CDCl_3$; 150 mg/mL; 120 scans; 20.115 MHz): δ(ppm) = 27.9 (3 × tBu-CH_3); 41.8 (Cys-C_β); 47.0 (Fmoc-C-9); 54.1 (Cys-C_α); 67.2 (Fmoc-CH_2-O); 83.0 (tBu-C_q); 119.9 (Fmoc-C-4,5); 125.1 (Fmoc-C-1,8); 127.6, 127.0 (Fmoc-C-3,2,6,7); 141.2 (Fmoc-C-4a,4b); 143.8, 143.7 (Fmoc-C-8a,9a); 155.7 (Fmoc-CO); 169.3 (Cys-CO).

TABLE 1

Assignments of the ^{13}C NMR signals of 3 ($CDCl_3$; 200 mg/mL; 20.115 MHz)

	3a	3b	3a/b
tBu-CH_3	27.9	27.9	27.9
S-glyceryl CH_2	35.5	35.4	35.4/35.5
Cys-C_β	36.2	36.3	36.2/36.3
Fmoc-C-9	47.1	47.0	47.0
Cys-C_α	54.6	54.5	54.5
S-glyceryl CH_2-OH	65.1	65.1	65.0/65.2
Fmoc-CH_2-O	67.2	67.2	67.1
S-glyceryl CH-OH	71.0	71.1	71.0 (2 ×)
tBu-C_q	83.0	82.9	82.9
Fmoc-C-4,5	120.0	119.9	119.9
Fmoc-C-1,8	125.1	125.1	125.1
Fmoc-C-3,2,6,7	127.1	127.1	127.0
Fmoc-C-3,2,6,7	127.7	127.7	127.7
Fmoc-C-4a,4b	141.2	141.2	141.2
Fmoc-C-8a,9a	143.7	143.7	143.7
Fmoc-CO	156.2	156.1	156.1
Cys-CO	169.9	169.9	169.3

N_α-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxypropyl]-[R]-cysteine tert.*-butyl ester (Fmoc-Dhc-OtBu)*, **3a**, **3b**, *and* **3a/b**

To a solution of **2** (1.92 g; 2.4 mmol) in dichloromethane (15 mL) zinc (1.1 g; 16.8 mmol) and a freshly prepared mixture of methanol, 32% hydrochloric acid (d = 1.16) and concentrated sulfuric acid (d = 1.84) (100 : 7 : 1; 8 mL) were added under vigorous stirring. After 15 min (S)-(-)-glycidol (1.78 g = 1.6 ml; 2.4 mmol) was added. The mixture was stirred for 5 h at 40°. The solvent was evaporated to about half of its original volume and diluted with 5% $KHSO_4$ (2 mL). This mixture was kept at −4° for 16 h and then extracted thrice with dichloromethane. The organic phase was dried over Na_2SO_4 and evaporated to dryness. **2** was obtained as a colorless oil. Yield of **3a**: 2.06 g (91%). R_f(I) = 0.72; R_f(II) = 0.47; R_f(IV) = 0.45; $C_{25}H_{31}NO_6S$ (473.6).

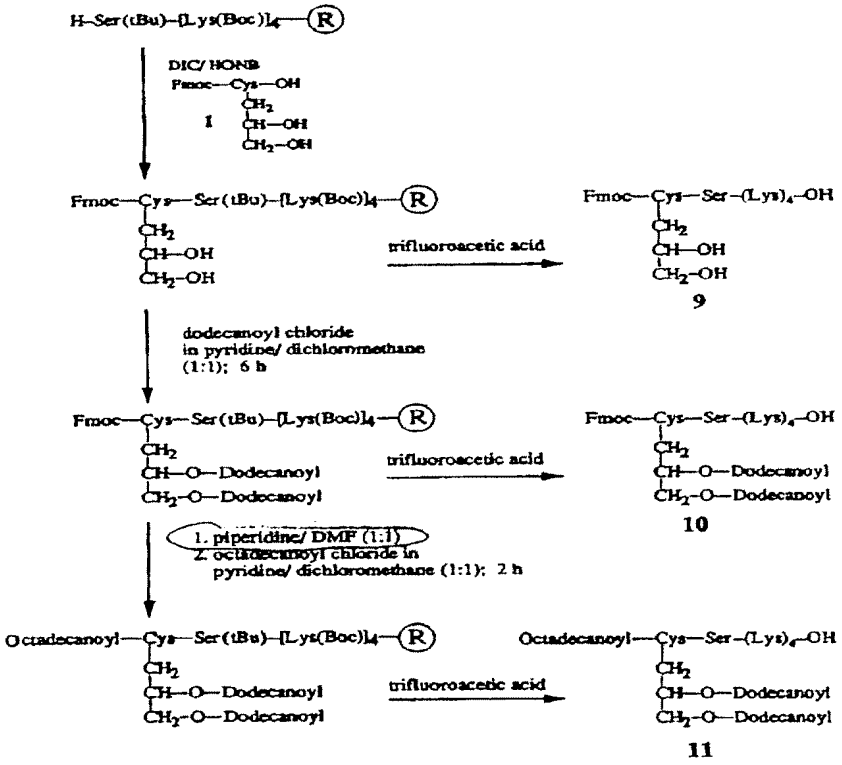
Analogously, **3b**, (yield: 1.93 g; 85%) and **3a/b** (yield: 1.84 g; 82%) were prepared by using (R)-(+)-glycidol and racemic glycidol, respectively. ^{13}C NMR: see Table 1 .

N_α-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxypropyl]-[R]-cysteine (Fmoc-Dhc-OH), **1a**, **1b**, *and* **1a/b** *(Procedure B)*

3a (400 mg, 0.84 mmol) was deprotected in trifluoroacetic acid (10 mL). The acid was removed *in vacuo* after 1 h. The oily product was dried over P_4O_{10}. Yield: 279 mg (79%). For analytical data see Procedure A.

Analogously, **1b** (Yield: 283 mg; 81%) and **1a/b** (Yield: 269 mg; 77%) were obtained after deprotection of **3b** and **3a/b**, respectively.

Fmoc-*S*-(2,3-dihydroxypropyl)-cysteine



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Synthesis of amphiphilic lipopeptides **9–11** by *O*- and *N*-acylation directly on the resin.

diphenylmethane (TDM reagent). Fmoc-containing peptides were additionally detected by UV (354 nm).

Analytical methods

The synthetic lipopeptides **8-11** were characterized by gas chromatography after total hydrolysis (6 N HCl, 110°, 18 h, in the presence of phenol), whereby the *S*-glycerylcysteinyl residue is partially decomposed (5). The obtained amino acids were automatically derivatized to the *N*-pentafluoropropionyl amino acid *n*-propyl esters. Their quantity as well as their optical purity was determined on a Chirasil-Val column (17) using enantiomer labeling (18). Optical rotations were measured in a 0.1 dm cell on a Perkin-Elmer 241 Spectropolarimeter. Field desorption mass spectra were recorded on a Varian MAT 711A (Varian, Bremen, FRG) at an ion source temperature of 50°. Ion spray mass spectra and ion spray tandem mass spectra were recorded on a Sciex API III triple-quadrupole mass spectrometer with 2400 Da mass range equipped with an ion spray ion source (Sciex, Toronto, Canada).

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N_a-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxy-(2R/2S)-propyl]-[R]-cysteine (Fmoc-Dhc-OH), **1 a/b** *(Procedure A)*

1 a/b was prepared according to a general procedure for the preparation of Fmoc-amino acids, which has been described previously (19): *S*-(2,3-dihydroxy-(2R/2S)-propyl)-[R]-cysteine (20) (820 mg; 4.2 mmol) was dissolved in 9% sodium carbonate (10 mL). A solution of fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (1.18 g; 3.5 mmol) in dioxane (10 mL) was added. The mixture was stirred for 2 h, then diluted with water (120 mL), and extracted with ethyl acetate (3 × 25 mL). The aqueous phase was acidified to pH 2 with concentrated hydrochloric acid. The aqueous phase was extracted with ethyl acetate (6 × 25 mL). The extract was washed with saturated sodium chloride solution (3 × 20 mL), water (3 × 20 mL), dried over sodium sulfate, and evaporated to dryness. Recrystallization from chloroform or dichloromethane at −20° yielded a colorless powder. m.p. 22°. Yield: 1.38 g (94%). $C_{21}H_{23}NO_6S$ (417.5). IS–MS: $[M + H]^+$ 418.5. R_f(I) = 0.16; R_f(II) = 0.04.

^{13}C NMR ($CDCl_3$; 200 mg/mL; 256 scans; 20.115 MHz): δ(ppm) = 35.1 (*S*-glyceryl CH_2); 35.6 (Cys-C_β); 47.0 (Fmoc-C-9); 53.8 (Cys-C_α); 65.0 (*S*-glyceryl CH_2-OH); 67.4 (Fmoc-CH_2-O); 71.3 (*S*-glyceryl CH-OH); 120.0 (Fmoc-C-4,5); 125.2 (Fmoc-C-1,8); 127.1 (Fmoc-C-3,2,6,7); 127.8 (Fmoc-C-3,2,6,7); 141.2 (Fmoc-C-4a,4b); 143.8, 143.6 (Fmoc-C-8a,9a); 156.4 (Fmoc-CO); 173.3 (Cys-CO).

N,N'-*Bis(fluorenylmethoxycarbonyl)-[R]-cystine-bis*-tert.-*butyl ester* (*(Fmoc-Cys-OtBu)$_2$*). **2**

L-Cystine bis-*tert.*-butyl ester (3.52 g; 10 mmol; refs. 5, 15, 21) and fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide ester (5.4 g; 16 mmol) were dissolved in tetrahydrofuran (10 mL). A solution of *N*-ethylmorpholine (2.55 g; 20 mmol) in tetrahydrofuran (5 mL) was added. After 3 h the mixture was evaporated to dryness. The residue was dissolved in ethyl acetate and washed three times with 5% $KHSO_4$ and water. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was recrystallized from dichloromethane/methanol (1 : 4; 300 mL) at −20°. The precipitate was washed twice with *tert.*-butanol/2-propanol (1 : 1) and dried over P_4O_{10}/KOH. Yield: 6.9 g (87%). R_f(I) = 0.89; R_f(II) = 0.81; R_f(IV) = 0.80; R_f(V) = 0.21. $C_{44}H_{48}N_2O_8S_2$ (797.0). IS–MS: $[M + H]^+$ 798. $[\alpha]_D^{20}$-6 (*c* 2, $CHCl_3$).

^{13}C NMR ($CDCl_3$; 150 mg/mL; 120 scans; 20.115 MHz): δ(ppm) = 27.9 (3 × tBu-CH_3); 41.8 (Cys-C_β); 47.0 (Fmoc-C-9); 54.1 (Cys-C_α); 67.2 (Fmoc-CH_2-O); 83.0 (tBu-C_q); 119.9 (Fmoc-C-4,5); 125.1 (Fmoc-C-1,8); 127.6, 127.0 (Fmoc-C-3,2,6,7); 141.2 (Fmoc-C-4a,4b); 143.8, 143.7 (Fmoc-C-8a,9a); 155.7 (Fmoc-CO); 169.3 (Cys-CO).

TABLE 1

Assignments of the ^{13}C NMR signals of 3 ($CDCl_3$; 200 mg/mL; 20.115 MHz)

	3a	3b	3a/b
tBu-CH_3	27.9	27.9	27.9
S-glyceryl CH_2	35.5	35.4	35.4/35.5
Cys-C_β	36.2	36.3	36.2/36.3
Fmoc-C-9	47.1	47.0	47.0
Cys-C_α	54.6	54.5	54.5
S-glyceryl CH_2-OH	65.1	65.1	65.0/65.2
Fmoc-CH_2-O	67.2	67.2	67.1
S-glyceryl CH-OH	71.0	71.1	71.0 (2 ×)
tBu-C_q	83.0	82.9	82.9
Fmoc-C-4,5	120.0	119.9	119.9
Fmoc-C-1,8	125.1	125.1	125.1
Fmoc-C-3,2,6,7	127.1	127.1	127.0
Fmoc-C-3,2,6,7	127.7	127.7	127.7
Fmoc-C-4a,4b	141.2	141.2	141.2
Fmoc-C-8a,9a	143.7	143.7	143.7
Fmoc-CO	156.2	156.1	156.1
Cys-CO	169.9	169.9	169.3

N_a-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxypropyl]-[R]-cysteine tert.-*butyl ester (Fmoc-Dhc-OtBu)*, **3a**, **3b**, *and* **3a/b**

To a solution of **2** (1.92 g; 2.4 mmol) in dichloromethane (15 mL) zinc (1.1 g; 16.8 mmol) and a freshly prepared mixture of methanol, 32% hydrochloric acid (d = 1.16) and concentrated sulfuric acid (d = 1.84) (100 : 7 : 1; 8 mL) were added under vigorous stirring. After 15 min (S)-(-)-glycidol (1.78 g = 1.6 mL; 2.4 mmol) was added. The mixture was stirred for 5 h at 40°. The solvent was evaporated to about half of its original volume and diluted with 5% $KHSO_4$ (2 mL). This mixture was kept at −4° for 16 h and then extracted thrice with dichloromethane. The organic phase was dried over Na_2SO_4 and evaporated to dryness. **2** was obtained as a colorless oil. Yield of **3a**: 2.06 g (91%). R_f(I) = 0.72; R_f(II) = 0.47; R_f(IV) = 0.45; $C_{25}H_{31}NO_6S$ (473.6).

Analogously, **3b**, (yield: 1.93 g; 85%) and **3a/b** (yield: 1.84 g; 82%) were prepared by using (R)-(+)-glycidol and racemic glycidol, respectively. ^{13}C NMR: see Table 1.

N_a-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxypropyl]-[R]-cysteine (Fmoc-Dhc-OH), **1a**, **1b**, *and* **1a/b** *(Procedure B)*

3a (400 mg, 0.84 mmol) was deprotected in trifluoroacetic acid (10 mL). The acid was removed *in vacuo* after 1 h. The oily product was dried over P_4O_{10}. Yield: 279 mg (79%). For analytical data see Procedure A.

Analogously, **1b** (Yield: 283 mg; 81%) and **1a/b** (Yield: 269 mg; 77%) were obtained after deprotection of **3b** and **3a/b**, respectively.

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The macrophage-activating lipopeptide-2 accelerates wound healing in diabetic mice

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Abstract: Wound healing in healthy individuals proceeds at an optimal rate. However, in patients, with – e.g.– locally impaired blood flow or diabetes, chronic wounds develop and often become infected. Chronic wounds mean a low quality of life for the afflicted patients, not to mention enormous costs. Rather than using recombinant growth factors to accelerate wound healing, we employed the toll-like receptor agonist macrophage-activating lipopeptide-2 (MALP-2) to improve the healing of full-thickness excision skin wounds in an animal model with obese, diabetic mice. A gene array experiment suggested that MALP-2 stimulates the release of various mediators involved in wound healing. Further data to be presented in this study will show (i) that MALP-2 is capable of stimulating the appearance of the monocyte chemoattractant protein-1 at the wound site, (ii) that this leads to increased leucocyte and, in particular, macrophage infiltration and (iii) that MALP-2-treated wounds closed 2 weeks earlier than vehicle-treated controls. MALP-2, thus, appears to stimulate the early inflammatory process needed to set in motion the ensuing consecutive natural steps of wound healing resulting in wound closure.

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Key words: chemokines – granulation tissue – inflammation – skin – wound healing

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Introduction

Wound healing proceeds in several stages. Haemostasis is followed after several hours by an inflammatory stage, during which leucocytes are recruited to clean the wound and provide various chemokines and growth factors. These, then, cause ingrowth of fibroblasts, formation of blood vessels, collagen deposition and epithelialization. During the last stage, which may take many months if not years, tissue remodelling and restructuring of scar tissue occur.

Wound healing in healthy persons proceeds at an optimal rate, and there is little need to interfere with this process. However, there are many cases when wounds fail to heal and become chronic. Among these are cases with perivascular disease presenting with leg ulcers, pressure sores or diabetic foot ulcers. Because the process of wound healing is so complex, a common cause for impaired healing is difficult to identify. However, ischaemia and diabetes predispose to poor wound healing, which may be further complicated by secondary infections.

Interestingly, a 'slight infection' is thought to accelerate wound healing (1). There is a certain logic in this notion, as inflammation is a wanted and natural phase in wound healing. Macrophages, being among the cells, which infiltrate a fresh wound during the inflammatory phase, are reportedly indispensable for this process (2). We know, now, that macrophages are the prime source of growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF)-A and -B and transforming growth factor (TGF)-β. Various growth factors are released within a certain time frame, different mediators being required at different times and concentrations for each healing phase. Various attempts were made to accelerate wound healing by isolated recombinant factors (3–10). Although some growth factors, such as, e.g., PDGF, have found their established place in wound management, their clinical use is limited by rapid clearance, by the need for a concerted action of several distinct growth factors to accomplish healing, and last not least, for economic reasons.

We want to report a novel approach, the use of the macrophage-activating lipopeptide-2 (MALP-2), to accelerate wound healing. We use full-thickness excision skin wounds in diabetic mice, an established animal model for impaired healing. MALP-2 was originally isolated from mycoplasmas, and is now synthetically available (11). The effects of MALP-2 are restricted to specific target cells expressing a combination of the toll-like receptors-2 and -6, which are required for signalling (12,13). Expression of toll-like receptor-2 has been extensively studied and it appears to be ubiquitously expressed by leucocytes (14,15), but only weakly by endothelial cells (16). However, both receptors are expressed and functional in at least two target cells important in the context of wound healing, macrophages and fibroblasts. As the name implies, MALP-2 is primarily a macrophage activator, but also fibroblasts can be activated by MALP-2 to release monocyte chemoattractant protein-1 (MCP-1), a chemokine, which attracts macrophages (13). Recently, mechanisms were described that explain how MALP-2 may be inactivated *in vivo* (13). Our data to be presented in this study show that MALP-2 is effectively accelerating wound closure in healing impaired diabetic mice. The underlying mechanism, which we discuss, is an initial stimulation of skin fibroblasts by MALP-2 to release MCP-1 and other chemokines. These chemokines first attract granulocytes, and later monocytes/macrophages to the wound site, where these latter cells are activated by remaining MALP-2 to release the growth factors required for accelerated wound healing.

Materials and methods

Malp-2

The biologically active enantiomer of MALP-2, S-[2,3-bis (palmitoyloxy)-(2*R*)-propyl]-cysteinyl-GNNDESNISFKEK, was synthesized and HPLC-purified as described, using (*R*)-(+)-oxiran-2-methanol (Fluka) as starting material for the lipid moiety (13,17). MALP-2 was kept as a stock solution of 1.3 mg/ml in 33% (v/v) 2-propanol/water at 4°C. The exact peptide content was determined using amino acid analysis and biological activity with the nitric oxide (NO) release assay. This assay was performed as described, with resident peritoneal exudate cells from C3H/HeJ LPS low-responder mice as a source of macrophages (11). C3H/HeJ mice were from Jackson Laboratories (Bar Harbor, ME, USA). One unit of macrophage-stimulating activity per millilitre is defined by the dilution yielding half-maximal stimulation. For the NO assay, MALP-2 was solubilized in 25 mM of octylglucoside in the first dilution step and then further diluted with medium. MALP-2 used in this study had a specific activity of 5×10^8 U/mg.

Gene array

Human dendritic precursor cells were thawed in a monocyte differentiation medium, as indicated by the manufacturer (Clonetics B BioWhittaker, Walkersville, MD, USA). Sealer protein (fibrinogen-B-containing solution) and thrombin from the Tisseel™ fibrin sealant kit were prepared according to the manufacturer's instructions (Baxter BioScience, Westlake Village, CA, USA). Briefly, 1.5-ml portions of the sealer protein at a concentration of 100 mg/ml was added to 10-cm culture plates (Becton Dickinson-Corning, Franklin Lakes, NJ, USA), and then mixed with 1.5 ml of thrombin solution at a concentration of 500 U/ml. After formation of the fibrin clots, they were overlaid with Tris-buffered saline and were stored at 4°C. Six plates were prepared – three with 1 ng/ml of MALP-2 and three negative controls without MALP-2. Dendritic precursor cells were added to the fibrin clots, and the fibrin clots were incubated at 37°C with 5% CO_2 in a Dendritic Precursor Cell Medium (Clonetics B BioWhittaker). After 2.5, 7 and 24 h, one plate from each group was washed with HBSS once, and then 3 ml of trypsin/EDTA was added for 10 min at 37°C. The detached cells were centrifuged, and the cell pellets were dipped in liquid nitrogen for 10 min. The samples were stored at −80°C and were processed for gene arrays using Clontech, examining gene arrays consisting of 1200 genes (human 1.2K-I). All data were compared to standard genes within the arrays, and the comparison within each group, experimental with MALP-2 versus untreated control, is based on these standard genes. Weak signals were eliminated following two criteria: (i) when the difference between the experiments and control was below or at the threshold of 1.69 and (ii) when the difference in spot intensity was below or at 3.

Wounding

Obese, diabetic, female mice – C57BLKS/Bom-db – were obtained from M&B (Ry, Denmark). BKS.Cg-m+/+Lepr^{db} were obtained from Jackson Laboratories (Bar Harbor). They were used for the wound healing experiments at an age between 12 and 15 weeks when they weighed 40–50 g. An area of approximately 4 × 4 cm of the dorsal skin of mice was shaved and depilated with water-soluble depilatory cream 'Veet sensitive plus' (Reckit Benckiser, Mannheim, Germany). After 3 min, the cream was carefully removed with citrate buffer (pH 6) followed by water. The skin was then briefly treated with Bepanthen® Lotion (Hoffmann-La Roche, Germany) to prevent skin irritation. Mice were left for at least 1 day before wounding. Wounding and changes of dressings were performed under a sterile work bench. The mice were anaesthetized with isoflurane using an anaesthetic vaporizer by Dräger, Lübeck, Germany. The skin was desinfected with Braunol® (Braun Melsungen, Germany) and local anaesthesia was performed using intracutaneous application of 30 µl of 2% Xylocain (Astra GmbH, Germany), which also prevents excessive bleeding. Braunol® was removed after 5 min, and the skin was defatted with trichloroethylene. An imprint of a circular 1.3-cm-diameter wound was made on the mid-back, and a full-thickness wound, including the panniculus carnosus, was created by excising this area. Smaller wounds of 0.8-cm diameter were created by a biopsy punch. Wounds were closed with a transparent film dressing (Hydrofilm®, Hartmann, Germany), which was made to firmly adhere by application of a thin film of special glue (Mastic-Verbandkleber, ASID Bonz und Sohn GmbH, Böblingen, Germany) around the edges. The transparent film dressing was further fixed with a second dressing (Fixomull®-stretch, Beiersdorf, Germany), containing a circular hole for observation of the wound. A volume of 70 µl of MALP-2 or vehicle control, respectively, (both containing 0.32% methylhydroxypropylcellulose and 5% mouse serum as carrier) was injected through the transparent film dressing into the wound bed at the indicated times. The puncture was closed with a small piece of film dressing. Mice were kept in groups of three in individual ventilation cages and were observed over a time period of at least 25 days. Routinely, the dressings were changed every fifth day or as required using ethyl acetate for removing the old ones. At these times, swabs were taken from the wounds and

were cultured in thioglycollate and on blood agar to monitor infections. Mice with infected wounds were omitted from the experiment. Wound closure was determined by tracing the wound diameter on transparent film. The marked areas were xerocopied, cut out and weighed to determine the surface area of the wound. Wound areas were expressed as percentage of the original wound. The animal experiments were approved by the county government of Braunschweig (Az 509.42502/07-05.99).

Determination of nucleic acids and hydroxyproline from skin samples

Samples from skin or wound tissue were delipidated using sequential washings with 0.5 ml each of methanol, methanol/chloroform (1 + 1) (v/v) and chloroform. After evaporation of the chloroform, samples were hydrolyzed in 10N HCl (37%) at 110°C overnight, taken to dryness over NaOH pellets and taken up in 350 µl of water. The hydrolysate was cleared using filtration through a 0.2-µm filter (Supror Acrodisc 13, Gelman). For determination of nucleic acids, the absorbancy of the filtrate was measured at 260 nm. Hydroxyproline was determined using Ehrlich's reagent as described (18,19).

Re-extraction and determination of MALP-2 and MCP-1 activity from the wound area after local application

Wounds were created on the mid-back of diabetic mice, and MALP-2 was applied as above. Mice were killed at different times after application of MALP-2 or vehicle control by administering an overdose of isoflurane. MALP-2 was recovered from the wound fluid and from the surrounding skin as follows. The wound-bed, still covered by film dressing, was washed once with 150 µl and twice with 100 µl of 25 mM of octylglucoside, 0.1% human serum albumin. The combined washings were heated for 5 min at 100°C and were centrifuged for 10 min at 11 000 × *g*, and supernatants were kept at −20°C until determination of MALP-2 by its macrophage-stimulating activity in the NO release assay. After removal of dressing, a 0.6-cm-wide ring of skin around the wound margin was excised. The excised skin was weighed, frozen in liquid nitrogen and pulverized in a mortar. Still in the cold, the homogenate was suspended in 1.5 ml of NaCl with 5% protease inhibitor for mammalian cell extracts (Sigma, Germany). This suspension was divided into two equal portions. One portion was mixed 1 + 1 with 50 mM of octylglucoside, heated and centrifuged as above. The supernatant was kept at −20°C, until determination of macrophage-stimulating activity. The other portion was centrifuged for 10 min at 11 000 × *g*, and the supernatant was kept at −20°C, until determination of MCP-1.

Histology and immunostaining

Histological evaluation was performed at various times after wounding. Mice were killed by administering an overdose of isoflurane, and 0.4-by-2-cm strips of the dorsal skin, including the wound area, were excised down to the fascia, embedded in GSV1 embedding medium (SLEE Technik GmbH Mainz, Germany) and snap-frozen in liquid nitrogen. Cryosectioning was performed at −21°C. Sections were fixed in acetone and were kept at −40°C, until stained with eosin-methylene blue (Wrigth's stain) or antibodies. Monoclonal antibodies used were BM8 (BMA Biomedicals, Augst, Switzerland), specific for macrophages, CD31, specific for endothelial cells (BD Biosciences, Pharmingen, USA), and a monoclonal antibody against the αV6 epitope of CD44, clone 9A4 (20) (a kind gift of M. Hegen and P. Herrlich, Forschungszentrum Karlsruhe, Institute of Toxicology and Genetics, Karlsruhe, Germany), to stain epidermal cells. Secondary antibodies were peroxidase-labelled or otherwise the APAAP staining procedure (Dako Diagnostika GmbH, Hamburg, Germany) was used according to the manufacturer's instructions.

Results

MALP-2-mediated gene activation in a primary human, monocyte-derived dendritic precursor cell line

MALP-2 was incorporated in Tisseel® fibrin sealant to approximate the conditions in a fresh wound. As expected, time-dependent upregulation was observed for precursor genes of tumour necrosis factor (TNF), interleukin-1 (IL-1) and IL-6, as well as for the chemokines MIP-1α, MIP-1β, RANTES and IL-8. In addition, genes coding for GM-CSF, VEGF, PDGF-A and -B, TGF-β and placenta growth factor were upregulated by MALP-2 at 2.5 and/or 7 h post-stimulation (Table 1). Although additional verification may be needed, the fact that upregulation of many genes was observed, the products of which had been previously identified in MALP-2-stimulated cultures of human or mouse cells, strengthens these preliminary data. They suggest that MALP-2 incorporated into fibrin clots of a wound can be expected to induce the expression of genes relevant to wound healing by infiltrating monocytes/macrophages.

MALP-2-stimulated leucocyte infiltration

The concept to use MALP-2 for an acceleration of wound healing rests on the ability of MALP-2 to attract and subsequently stimulate leucocytes, particularly macrophages. We had previously shown that skin as well as lung fibroblasts upon stimulation with MALP-2 are able to liberate as much MCP-1 on a per-cell basis as macrophages (13). Fibroblasts isolated from diabetic mice were equally responsive (not shown). It was, therefore, reasonable to assume that intracutaneous application of MALP-2 would, through stimulation of skin fibroblasts or macrophages, result in local leucocyte infiltration. A pilot experiment in diabetic mice showed that already 0.5–1 µg of MALP-2 led to a significant leucocyte infiltration, which reached a maximum after day 3. These data were obtained by determining nucleic acid from biopsy punches as measure of cell numbers and were supported by visual observation (not shown). Next, full-thickness excision wounds of 1.3-cm diameter were made on the backs of diabetic mice, covered by film dressing, and a single dose of 0.5 µg of MALP-2 or vehicle control was injected through the dressing into the fresh wound bed. Mice were

Table 1. Selection of macrophage-activating lipopeptide-2 (MALP-2) inducible genes in human monocytes, which are possibly relevant to wound healing

Gene or precursor gene	Gene product	Function	Gene regulation by MALP-2[1] after 2.5 h	7 h	24 h
α-1-antitrypsin		Protease inhibitor	b.t.[2]	b.t.	1.7
Cathepsin H		Protease	-2.7	-2.1	1.9
ENA-78	Epithelial neutrophil-activating protein-78	Chemokine	b.t.	2.7	3
G-CSF	Granulocyte colony-stimulating factor	Growth factor	3.5	b.t.	b.t.
GM-CSF	Granulocyte-macrophage colony-stimulating factor	Growth factor	3.5	b.t.	b.t.
IL-1	Interleukin-1	Pro-inflammatory cytokine, multiple functions, endogenous pyrogen	2.7	6.1	b.t.
IL-6	Interleukin-6	Pro-inflammatory cytokine, multiple functions	2	9.4	1.7
IL-8	Interleukin-8	Chemokine, attracting granulocytes	1.8	4.1	b.t.
LEI	Leucocyte elastase inhibitor	Protease inhibitor	b.t.	1.8	-1.8
LIF	Leukaemia inhibitory factor	Involved in nerve repair	2.9	b.t.	b.t.
MIF-related protein-14		Involved in leucocyte adhesion	b.t.	b.t.	1.8
MIP-1α	Macrophage inflammatory protein	Chemokine, attracting leucocytes	b.t.	4.2	2.8
MIP-1β	Macrophage inflammatory protein	Chemokine, attracting leucocytes	1.7	3.5	2.1
MIP-2α	Macrophage inflammatory protein	Chemokine, attracting neutrophils	b.t.	5.1	4.3
MMP-11	Matrix metalloproteinase-11	Protease	b.t.	2.1	b.t.
MMP-12	Matrix metalloproteinase-12	Protease	b.t.	3.5	b.t.
PDGF-A	Platelet-derived growth factor-A	Growth factor for connective tissue cells	b.t.	1.7	b.t.
PDGF-B	Platelet-derived growth factor-B	Growth factor for connective tissue cells	1.8	b.t.	-2
Placenta growth factor		Growth factor involved in angiogenesis	2.2	4.9	
RANTES	Regulated upon activation, normal T cell expressed and secreted	Chemokine, attracting monocytes, Memory T cells and eosinophils	2.6	b.t.	b.t.
SCYA1		MIP-related chemokine	b.t.	5	b.t.
TGF-β2	Transforming growth factor	Induction of collagen synthesis	2.2	b.t.	-1.9
TIMP-1		Protease inhibitor	b.t.	1.8	1.9
TNF-α	Tumour necrosis factor	Pro-inflammatory cytokine	2.8	1.7	-4.7
VEGF	Vascular endothelial growth factor	Growth factor involved in angiogenesis	1.8	1.8	b.t.

[1]Activation (x-fold), compared to unstimulated control cells. Negative values indicate downregulation.
[2]Below threshold.

killed 1, 3, 7 and 13 days after MALP-2 application, and cryosections were stained with Wright's stain or monoclonal antibodies detecting macrophages (BM8), keratinocytes of the epidermis (CD44) or endothelial cells (CD31), respectively. Granulocytes appeared early and also in wounds treated with control vehicle, but were further increased in MALP-2-treated wounds on day 1 post-wounding (Fig. 1, Table 2). PMNs started to disappear on day 3 and were negligible on day 7. In contrast, MALP-2-treated wounds showed a marked macrophage infiltration, which started on day 1 post-wounding, reached a maximum around day 3 and lasted at least until day 7 (Fig. 1, Table 2). In fact, monocyte/macrophage numbers were so dense on day 3 that they were impossible to count in the area of most intense infiltration, i.e. below the panniculus near the wound edge (Fig. 1). Consequently, a semiquantitative enumeration of the BM8-positive cells at this time was only feasible in the subcutaneous fat cell layer (Table 2).

On day 7, a thickening of the CD44-positive epidermis was observed, indicating proliferating and migrating keratinocytes (24). This CD44-positive zone extended to about 0.8 cm around MALP-2-treated wounds and lasted until at least day 13, whereas untreated control wounds showed a CD44-positive margin of maximally 0.2 cm (Fig. 2). Differences in staining of CD31-positive endothelium became noticeable on day 7, but were distinctly enhanced near the wound edge of MALP-2-treated wounds on day 13 (Fig. 2).

Fate of MALP-2, when applied to skin wounds

In order to answer the question whether MALP-2, when applied to a fresh wound, would persist there for a sufficient time span to be effective, an experiment was designed to retrieve MALP-2 from wound fluid and wound margin of diabetic mice, and to simultaneously determine MCP-1 from the wound tissue at various times after MALP-2

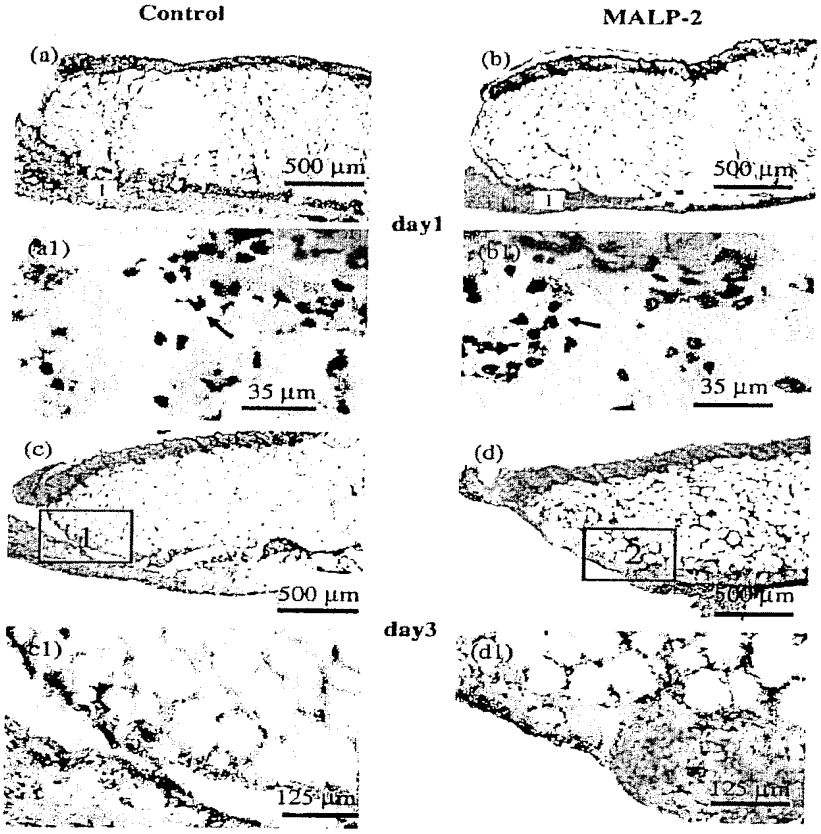

Figure 1. Infiltration of leucocytes in macrophage-activating lipopeptide-2 (MALP-2)-treated versus control wounds. Full-thickness circular wounds of 1.3-cm diameter were made on the dorsal skin of diabetic mice, closed with transparent film dressing, and 70 µl volumes containing 0.5 µg of MALP-2 or vehicle control, respectively, were injected into the wound bed. Overviews are shown of cryosections stained with Wright's stain from near the wound margin of control (a) and MALP-treated (b) wounds, 1 day post-wounding. Infiltration of mainly PMN (horse shoe or ring-shaped, see arrows) and a few macrophages is shown at higher magnification of the small rectangular areas (a1 and b1, respectively). (c) Overview of a 3-day-old wound treated with control vehicle, stained with macrophage/monocyte-specific BM8 monoclonal antibodies in red (APAAP technique); c1 shows area 1 at higher magnification. (d) Overview of a 3-day-old MALP-2-treated wound stained with BM8 monoclonal antibodies; d1 shows area 2 at higher magnification. Note the intense staining of the BM8-positive infiltrate

Table 2. Infiltration of PMN and BM8-positive monocytes/macrophages in control- and macrophage-activating lipopeptide-2 (MALP-2)-treated wounds

	PMN[1]		BM8-positive monocytes/macrophages[2]	
Day	Control	MALP-2-treated	Control	MALP-2-treated
1	51 ± 17	120 ± 68[3]	29 ± 15	49 ± 13[3]
3	21 ± 12	19 ± 9	8 ± 6	71 ± 18[3]
7	3 ± 2	4 ± 2	25 ± 12	47 ± 17[3]

[1]PMN were counted at ×40 magnification by two independent observers in three squares of 312-µm lengths in two different cryosections from different wounds. Cells were counted close to the wound edge next to the area indicated by the rectangle in Fig. 1.
[2]BM8-positive cells were counted in corresponding areas of neighbouring cryosections except on day 3, when the cells were too dense to be evaluated in this area and were counted in the fat cell layer. Data are the means of six counts ± SD.
[3]Significant differences as calculated by Student's *t*-test between cell counts in MALP-treated versus control wounds ($P < 0.05$).

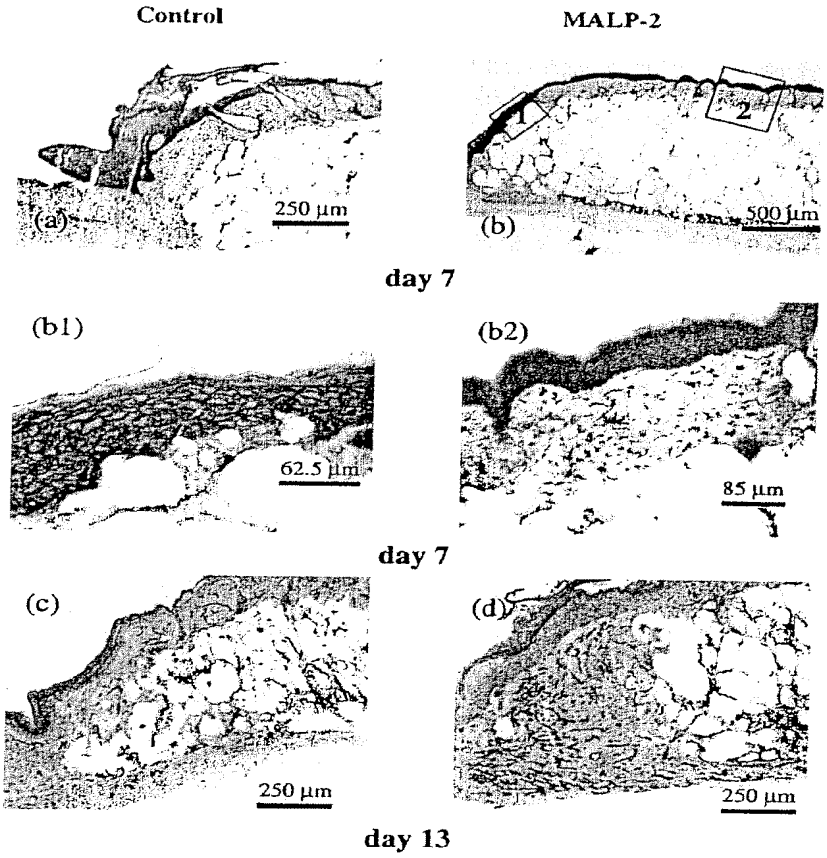

Figure 2. Expression of CD44 and CD31 in macrophage-activating lipopeptide-2 (MALP-2)-treated versus control wounds. Wounds were applied and treated as in Fig. 1. Active keratinocytes are stained with CD44-specific monoclonal antibodies, indicating proliferating and migrating keratinocytes (dark brown peroxidase stain). (a) Overview of a control wound near the wound edge on day 7 stained with CD44 monoclonal antibodies. (b) The corresponding area from an MALP-2-treated wound. Note the CD44 stain extending far away from the wound edge to the left. (b1 and b2) Areas 1 and 2 at higher magnification, showing the layers of CD44-positive keratinocytes. (c) CD31-stained endothelial cells in a control wound 13 days post-wounding. (d) The corresponding area from an MALP-2-treated wound on day 13 post-wounding. Note the massive neovascularization in the MALP-2-treated wound

application. Full-thickness excision wounds of 1.3-cm diameter were made on the backs of diabetic mice, covered with film dressing, and were treated with a single dose of 0.5 µg of MALP-2, which was injected through the dressing into the wound bed. Mice were killed after various times, and the remaining biological activity of MALP-2 was determined in the wound bed and the surrounding tissue with the NO release assay. This test was negative with samples from untreated control wounds. As shown in Table 3, most MALP-2 activity disappeared from the wound bed overnight, but about 25% could still be retrieved from the surrounding tissue after 24 h.

Appearance of MCP-1 in the wound bed after MALP-2 application

According to the above data, there would be ample time to allow MALP-2 to efficiently stimulate cells. As an example for such stimulation, we determined the chemokine MCP-1 in the wound bed (groups of three mice, see above). The remainder of the wound samples that had served for MALP-2 determination served to measure MCP-1 using ELISA technique. MCP-1 was assayed at [18] the time points 1, 3, 5, 7, 18 and 24 h. Wounds stimulated with 0.5 µg of MALP-2 showed a peak of MCP-1 (167.8 ± 20.0 pg/mg of skin) at 7 h, whereas control wounds contained only

Table 3. Recovery of biologically active macrophage-activating lipopeptide-2 (MALP-2) from wound bed and surrounding tissue after various times as determined by the nitric oxide (NO) release assay[1]

Time (h)	NO-release Wound fluid + lavage (%)	NO-release Skin (%)	NO-release Total (%)
1	ø 25 ± 12	ø 49 ± 15	ø 75 ± 8
3	ø 17 ± 9	ø 32 ± 14	ø 49 ± 6
5	ø 13 ± 5	ø 29 ± 16	ø 42 ± 18
7	ø 11 ± 3	ø 19 ± 6	ø 30 ± 4
18	ø 3.0 ± 1.0	ø 21 ± 16	ø 24 ± 17
24	ø 3.1 ± 1.8	ø 28 ± 8	ø 31 ± 10

[1]0.5 µg of MALP-2, corresponding to 175 kU (100%), was applied to the film-covered wound bed. Wound beds were washed, and a 6-mm ring around the wound was excised after the indicated times. MALP-2 was extracted and determined as in the section entitled 'Materials and methods'. Values for each time point are the average from groups of three animals ± SD.

43.6 ± 5.8 pg/mg of skin at this time. MCP-1 levels fell to 76.0 ± 8.5 pg/mg of skin at 18 and 24 h, control wound showing 18.4 ± 1.1 pg/mg of skin at this time. Thus MCP-1 was significantly elevated in MALP-2-treated wounds ($P < 0.001$).

Dose-dependent, MALP-2-induced increase in cell and collagen content of skin wounds

In order to find an approximate value for an appropriate MALP-2 dose in fresh wounds, full-thickness excision wounds of 0.8-cm diameter were made on the backs of diabetic mice (groups of four animals), covered, and were treated with a single dose of MALP-2 as above. Visual observation revealed leucocytes in the wound fluid after about 5 days, with doses of 2.5 µg of MALP-2 and higher. A cursory examination indicated that these consisted initially of mainly PMNs, followed later by macrophages. The mice were killed 10 days after wounding, and newly formed tissue of the initial area of 0.8-cm diameter was retrieved from the wound area, and were analysed for nucleic acid content as a measure of cell number and collagen as an indicator of wound healing. Although the values were somewhat scattered due to technical difficulties in reproducibly removing the newly formed tissue from the wound, a single dose of 5 µg of MALP-2 appeared optimal, yielding 72 ± 24 µg of hydroxyproline per wound versus 46 ± 7 µg in the vehicle-control-treated specimen ($P < 0.085$) and 972 ± 75 µg of nucleic acid versus 405 ± 63 µg ($P < 0.001$), respectively.

MALP-2-dependent acceleration of wound closure in diabetic mice

Based on the above results, the effects of MALP-2 on wound healing were tested. Because full-thickness excision wounds of small-diameter, possibly through wound contraction, tended to close rapidly, thus making the effects of MALP-2 less conspicuous, we chose to study the effects of MALP-2 on rather large, full-thickness excision wounds of 1.3-cm diameter. These wounds were again covered with transparent film dressing. MALP-2-dependent histological changes on the one hand and MALP-2-mediated collagen deposition on the other hand had suggested a dose range but not given a clear indication of an optimal MALP-2 dose for wound healing. Therefore, MALP-2 doses between 0.05 and 5 µg were injected through the dressing into the wound bed at time intervals of a few days. All these doses, when applied between day 0 and day 5, accelerated wound healing. Usually around day 5, MALP-2-treated wounds, but not the vehicle controls, showed a distinctly yellowish exudate, indicating the appearance of leucocytes. The results

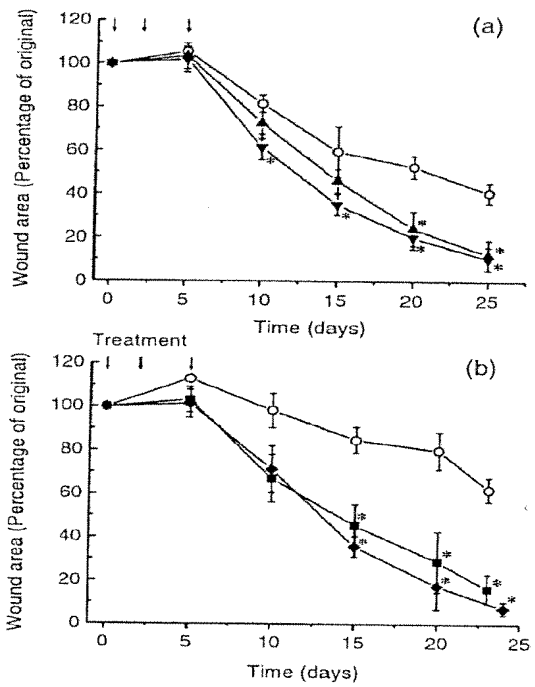

Figure 3. Accelerated wound closure by macrophage-activating lipopeptide-2 (MALP-2) treatment. Full-thickness circular wounds of 1.3-cm diameter were made on the dorsal skin of diabetic mice, closed with a film dressing and treated on days 0, 2 and 5 with the indicated doses of MALP-2 or vehicle control as in Fig. 1. Wound areas are expressed as percentage of the original wound. Group sizes are indicated for the various doses, and values are mean ± SD. Vehicle control (O, a, $n = 3$; b, $n = 4$); 2.5 µg of MALP-2: (▲, $n = 9$); 0.5 µg of MALP-2: (▼, $n = 8$); 0.1 µg of MALP-2: (◆, $n = 5$), or 0.05 µg of MALP-2: (■, $n = 6$). * significantly different ($P < 0.001$) from respective vehicle control, as calculated by Student's t-test

from two different experiments with different doses are shown in Fig. 3. Although healing of control groups varied with the batches of animals used, differences between the areas of MALP-2-treated and respective vehicle control wounds became significant around day 10. MALP-2-treated wounds closed at days 35 ± 5, whereas control wounds needed 50 ± 5 days to heal. A fourth MALP-2 application on day 13 did not further accelerate wound closure.

In another, long-term, experiment, healed MALP-2-treated and control wounds were compared. On the shaven backs of mice that were wounded 87 days previously, complete regeneration of the skin, including hair growth, was observed, except for a small, oval, hairless area along the dorsal midline. One interesting and unexpected observation, which needs further confirmation, was the distinctly better re-growth of fur around MALP-2-treated versus control wounds. The histological appearance of these wounds that had closed for 1 or 2 weeks was indistinguishable, regardless of whether these wounds were MALP-treated or controls (not shown).

In summary, these observations demonstrate that healed MALP-2-treated wounds were not qualitatively different from closed control wounds and were equally stable, but healing was accelerated by 2 weeks.

Discussion

Macrophages, being the source of several growth factors and other mediators, were earlier found to be essentially involved in the inflammatory phase of wound healing (2,25). Our data suggest that the topical application of MALP-2 to accelerate healing by recruiting and activating macrophages may be an attractive and economic alternative to the use of single recombinant growth factors for the treatment of chronic wounds, when the cause of impaired healing lies in the lack of a sufficient trigger to initiate the healing process. This appears to be the case in the healing-impaired diabetic mouse. Whether MALP-2 is beneficial to the treatment of human chronic wounds will have to await a phase-2 study. It is to be expected that MALP-2 will only be useful, when applied to carefully debrided, non-inflamed wounds.

MALP-2 by itself has no chemotactic activity (26) and, in contrast to its parent molecule MALP-404 or M161Ag, respectively (27), it does not activate complement, generating complement-derived chemotactic peptides (28). However, we have previously shown that MALP-2 can stimulate embryonic fibroblasts to release MCP-1 (13), a chemokine attracting monocytes/macrophages. Similar data were obtained with skin fibroblasts isolated from diabetic mice (Mühlradt, unpublished). As shown in this study, sufficient MALP-2 remained in the wound tissue after a single application to stimulate monocytes/macrophages immigrating at later times (Table 3). Data from the DNA array study, although of a preliminary nature, and from previous publications (22,23) suggest that MALP-2-stimulated monocytes/macrophages release a mixture of mediators and growth factors relevant to wound healing. The release of pro-inflammatory cytokines, such as TNF in particular, may cause undesired side effects. However, release of TNF is early and of transient nature (Table 1). Moreover, the organism becomes refractory to repeated MALP-2 stimulation, as far as TNF release is concerned, whereas release of chemokines, such as MCP-1 and MIP-1, is unaffected by repeated MALP-2 application ((22); Deiters, unpublished). IL-1 release, on the other hand, may be beneficial through induction of IL-8, rather than harmful to wound healing (29).

A rapid epithelialization is of great importance to prevent secondary infections. We have, so far, no *in vitro* data, which indicate a direct action of MALP-2 on isolated keratinocytes. However, the thickening of the epidermis in proximity to MALP-2-treated wounds and the high level of expression of the hyaluronate cell surface receptor CD44 by newly formed epithelium (Fig. 2) suggest at least an indirect MALP-2 effect on keratinocytes. Studies by Oksala et al. (30) and Kaya et al. (24) have shown that migrating and proliferating keratinocytes express high levels of CD44. Significantly, Yu and Stamenkovic were able to correlate expression of CD44 with binding of matrix metalloproteinase-9 and cleavage of latent TGF-β by this enzyme (31). This process may well be stimulated by MALP-2.

Recruitment and stimulation of macrophages by various preparations have earlier been used by other groups to accelerate wound healing in different animal models (32–35). Thus, early pilot experiments by Leibovich and Danon showed glucan-mediated accelerated wound healing in mice (32). Later, Laato et al. reported that small numbers of live *Staphylococcus aureus* improved collagen deposition and formation of granulation tissue, which was associated with increased numbers of macrophages (1). More recently, live *S. aureus* were replaced by topical application of peptidoglycan from these bacteria in a different model of wound healing (34). Peptidoglycan is a rather complex macromolecule, difficult to purify and not accessible by synthesis. In contrast, MALP-2 is a well-defined,

synthetically available, highly purified substance with equally well-defined receptors (12,13), known mechanisms of inactivation (13), and traceable *in situ* activity (Table 3).

Another very potent macrophage activator, but not applicable to human use, is, of course, LPS endotoxin. *In vitro* effects of MALP-2 and endotoxin are very similar, in spite of signalling through different toll-like receptors. However, important differences exist, when these substances are administered *in vivo*: MALP-2, when intravenously injected, is about 1000 times less pyrogenic in the rabbit on a weight basis than LPS (36), and MALP-2 is likely to be *in situ* inactivated by leucocytes infiltrating the MALP-2-treated wound by at least two mechanisms, deacylation and oxidation of the thioether group (13), whereas LPS, after binding to a specific serum protein is transported to the liver and persists there in an active form (37).

When discussing the mechanism of MALP-2 effects on wound healing, one has to realize that the process of wound healing is too complex to be reduced to a few simple steps. Many different cell populations interact with one another and the extracellular matrix in a complicated interplay within time and space. Interactions occur through direct cell-to-cell contact, primary and secondary liberation of soluble mediators and the regulation of the expression of receptors for these mediators. Yet, the combination of our *in vitro and in vivo* data allows us to propose a simplified possible mechanism of how MALP-2 contributes to accelerated wound healing in the following steps: (i) MALP-2, when applied to the wounded skin, stimulates fibroblasts and a few skin macrophages to liberate chemokines, as shown in this study for MCP-1, (ii) MCP-1 and further chemotactically active mediators cause an increased local infiltration of first neutrophils, later monocytes/macrophages (Fig. 1), (iii) these macrophages and possibly other yet unidentified contributing cells will then be stimulated by residual small amounts of MALP-2 deposited at the wound site to release various growth factors, as is suggested by our gene array experiment (Table 1), and leading, among other effects, to enhanced neovascularization (Fig. 2), (iv) excess of MALP-2 is degraded by infiltrating leucocytes by the above mentioned mechanisms (13) and (v) once the natural chain of events is set in motion, the reparative processes following the inflammatory stage result in wound closure, which does not require additional stimulation to proceed.

In conclusion, the present study shows that MALP-2 appears to stimulate wound healing in the initial inflammatory phase, triggering the natural cause of wound repair and resulting in normal restructured skin without hypertrophic growth of granulation tissue.

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We want to report a novel approach, the use of the macrophage-activating lipopeptide-2 (MALP-2), to accelerate wound healing. We use full-thickness excision skin wounds in diabetic mice, an established animal model for impaired healing. MALP-2 was originally isolated from mycoplasmas, and is now synthetically available (11). The effects of MALP-2 are restricted to specific target cells expressing a combination of the toll-like receptors-2 and -6, which are required for signalling (12,13). Expression of toll-like receptor-2 has been extensively studied and it appears to be ubiquitously expressed by leucocytes (14,15), but only weakly by endothelial cells (16). However, both receptors are expressed and functional in at least two target cells important in the context of wound healing, macrophages and fibroblasts. As the name implies, MALP-2 is primarily a macrophage activator, but also fibroblasts can be activated by MALP-2 to release monocyte chemoattractant protein-1 (MCP-1), a chemokine, which attracts macrophages (13). Recently, mechanisms were described that explain how MALP-2 may be inactivated *in vivo* (13). Our data to be presented in this study show that MALP-2 is effectively accelerating wound closure in healing impaired diabetic mice. The underlying mechanism, which we discuss, is an initial stimulation of skin fibroblasts by MALP-2 to release MCP-1 and other chemokines. These chemokines first attract granulocytes, and later monocytes/macrophages to the wound site, where these latter cells are activated by remaining MALP-2 to release the growth factors required for accelerated wound healing.

Materials and methods

Malp-2

The biologically active enantiomer of MALP-2, S-[2,3-bis (palmitoyloxy)-(2*R*)-propyl]-cysteinyl-GNNDESNISFKEK, was synthesized and HPLC-purified as described, using (*R*)-(+)-oxiran-2-methanol (Fluka) as starting material for the lipid moiety (13,17). MALP-2 was kept as a stock solution of 1.3 mg/ml in 33% (v/v) 2-propanol/water at 4°C. The exact peptide content was determined using amino acid analysis and biological activity with the nitric oxide (NO) release assay. This assay was performed as described, with resident peritoneal exudate cells from C3H/HeJ LPS low-responder mice as a source of macrophages (11). C3H/HeJ mice were from Jackson Laboratories (Bar Harbor, ME, USA). One unit of macrophage-stimulating activity per millilitre is defined by the dilution yielding half-maximal stimulation. For the NO assay, MALP-2 was solubilized in 25 mM of octylglucoside in the first dilution step and then further diluted with medium. MALP-2 used in this study had a specific activity of 5×10^8 U/mg.

Gene array

Human dendritic precursor cells were thawed in a monocyte differentiation medium, as indicated by the manufacturer (Clonetics B BioWhittaker, Walkersville, MD, USA). Sealer protein (fibrinogen-B-containing solution) and thrombin from the Tissel™ fibrin sealant kit were prepared according to the manufacturer's instructions (Baxter BioScience, Westlake Village, CA, USA). Briefly, 1.5-ml portions of the sealer protein at a concentration of 100 mg/ml was added to 10-cm culture plates (Becton Dickinson-Corning, Franklin Lakes, NJ, USA), and then mixed with 1.5 ml of thrombin solution at a concentration of 500 U/ml. After formation of the fibrin clots, they were overlaid with Tris-buffered saline and were stored at 4°C. Six plates were prepared – three with 1 ng/ml of MALP-2 and three negative controls without MALP-2. Dendritic precursor cells were added to the fibrin clots, and the fibrin clots were incubated at 37°C with 5% CO_2 in a Dendritic Precursor Cell Medium (Clonetics B BioWhittaker). After 2.5, 7 and 24 h, one plate from each group was washed with HBSS once, and then 3 ml of trypsin/EDTA was added for 10 min at 37°C. The detached cells were centrifuged, and the cell pellets were dipped in liquid nitrogen for 10 min. The samples were stored at −80°C and were processed for gene arrays using Clontech, examining gene arrays consisting of 1200 genes (human 1.2K-I). All data were compared to standard genes within the arrays, and the comparison within each group, experimental with MALP-2 versus untreated control, is based on these standard genes. Weak signals were eliminated following two criteria: (*i*) when the difference between the experiments and control was below or at the threshold of 1.69 and (*ii*) when the difference in spot intensity was below or at 3.

Wounding

Obese, diabetic, female mice – C57BLKS/Bom-db – were obtained from M&B (Ry, Denmark). BKS.Cg-m+/+Lepr^{db} were obtained from Jackson Laboratories (Bar Harbor). They were used for the wound healing experiments at an age between 12 and 15 weeks when they weighed 40–50 g. An area of approximately 4 × 4 cm of the dorsal skin of mice was shaved and depilated with water-soluble depilatory cream 'Veet sensitive plus' (Reckit Benckiser, Mannheim, Germany). After 3 min, the cream was carefully removed with citrate buffer (pH 6) followed by water. The skin was then briefly treated with Bepanthen™ Lotion (Hoffmann-La Roche, Germany) to prevent skin irritation. Mice were left for at least 1 day before wounding. Wounding and changes of dressings were performed under a sterile work bench. The mice were anaesthetized with isoflurane using an anaesthetic vaporizer by Dräger, Lübeck, Germany. The skin was desinfected with Braunol™ (Braun Melsungen, Germany) and local anaesthesia was performed using intracutaneous application of 30 µl of 2% Xylocain (Astra GmbH, Germany), which also prevents excessive bleeding. Braunol™ was removed after 5 min, and the skin was defatted with trichloroethylene. An imprint of a circular 1.3-cm-diameter wound was made on the mid-back, and a full-thickness wound, including the panniculus carnosus, was created by excising this area. Smaller wounds of 0.8-cm diameter were created by a biopsy punch. Wounds were closed with a transparent film dressing (Hydrofilm™, Hartmann, Germany), which was made to firmly adhere by application of a thin film of special glue (Mastic-Verbandkleber, ASID Bonz und Sohn GmbH, Böblingen, Germany) around the edges. The transparent film dressing was further fixed with a second dressing (Fixomull™-stretch, Beiersdorf, Germany), containing a circular hole for observation of the wound. A volume of 70 µl of MALP-2 or vehicle control, respectively, (both containing 0.32% methylhydroxypropylcellulose and 5% mouse serum as carrier) was injected through the transparent film dressing into the wound bed at the indicated times. The puncture was closed with a small piece of film dressing. Mice were kept in groups of three in individual ventilation cages and were observed over a time period of at least 25 days. Routinely, the dressings were changed every fifth day or as required using ethyl acetate for removing the old ones. At these times, swabs were taken from the wounds and

Differential recognition of structural details of bacterial lipopeptides by toll-like receptors

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The question which detailed structures of bacterial modulins determine their relative biological activity and respective host cell receptors was examined with synthetic variants of mycoplasmal lipopeptides as model compounds, as well as recombinant outer surface protein A (OspA) of *Borrelia burgdorferi* and lipoteichoic acid. Mouse fibroblasts bearing genetic deletions of various toll-like receptors (TLR) were the indicator cells to study receptor requirements, primary macrophages served to measure dose response. The following results were obtained: (i) the TLR system discriminates between modulins with three and those with two long-chain fatty acids in their lipid moiety, in that lipopeptides with three fatty acids were recognized by TLR2, whereas those with two long-chain fatty acids and lipoteichoic acid required the additional cooperation with TLR6; (ii) substitution of the free N terminus of mycoplasmal lipopeptides with an acetyl or palmitoyl group decreased the specific activity; (iii) removal of one or both ester-bound fatty acids lowered the specific activity by five orders of magnitude or deleted biological activity; (iv) oxidation of the thioether group lowered the specific activity by at least four orders of magnitude. The implications of these findings for physiological inactivation of lipopeptides and host-bacteria interactions in general are discussed.

Key words: Bacterial lipoprotein / Inflammation / Toll-like receptor / Macrophage / Mycoplasma

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1 Introduction

The innate immune system reacts to stimuli from invading microorganisms by applying immediate defense mechanisms such as phagocytosis, killing through activated oxygen and nitrogen species [1] and proteolysis [2], and by alerting and attracting cells of the specific immune system through the action of cytokines and chemokines. The initial recognition of microorganisms is mediated by a set of germ-line-encoded receptors – pattern recognition receptors or toll-like receptors (reviewed in [3, 4]) – with specificities for conserved molecular structures, so-called modulins [5], shared by a large group of pathogens. Typical modulins are compounds associated with either gram-negative bacteria, such as LPS, or gram-positive bacteria, such as lipoteichoic acid (LTA), or other bacterial structures, such as peptidoglycan. Last not least, bacterial lipoproteins and -peptides have long been known to be immunostimulatory compounds [6], in particular strong Mφ activators [7–9]. They are the most common modulins being present in both gram-negatives and gram-positives, and found even in wall-less bacteria such as mycoplasmas and ureaplasmas (reviewed in [10, 11]).

Members of the toll receptor family are expressed differentially among host cells and appear to specifically respond to different modulins. Thus, whereas TLR4 responds to LPS [12, 13], TLR2 mainly interact with bacterial lipoproteins [14–17]. However, some of these lipoproteins require the cooperation of TLR2 with TLR6 for signaling [18–20]. Among these latter are lipopeptides from mycoplasmas, *e.g.* the Mφ-activating lipopeptide

[I 23312]

Abbreviations: LTA: Lipoteichoic acid **MALP-2:** Macrophage-activating lipopeptide of 2 kDa **MCP-1:** Monocyte chemoattractant protein -1 **MDP-OspA:** Delipidated form of OspA **Lip-OspA:** Outer surface lipoprotein A **TLR:** Toll-like receptor

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of 2 kDa (MALP-2). Reports on receptor usage of LTA are conflicting [16, 21].

Bacterial lipoproteins are N-terminally modified by a diacyloxypropyl group which is bound with a thioether linkage to the N-terminal cysteine. This N-terminal cysteine is commonly substituted by a third long-chain fatty acid through an amide bond blocking the N terminus [10]. Lipopeptides from *Mycoplasma fermentans*, *Mycoplasma hyorhinis* and possibly many other mycoplasma species lack this third fatty acid (see Fig. 1) [9, 22]. Such diacylated lipopeptides are particularly potent, comparable to LPS, in stimulating monocytes/Mϕ at pM concentrations [8, 9, 22].

The aim of the present study was to identify the molecular details in the structures of bacterial lipopeptides which determine their recognition by individual TLR as well as their relative biological activity, and possible mechanisms of inactivation in the host. To this end variants of the mycoplasmal lipopeptide MALP-2 sharing an identical peptide moiety but substituted with different lipid moieties, or with identical lipid moiety but different peptides, or an oxidized species of MALP-2 were synthesized. These compounds were compared with LTA and recombinant tri-lipidated lipopeptide from *B. burgdorferi* for their potential to induce nitric oxide or chemokine release, respectively, in primary Mϕ from normal mice or primary mouse fibroblasts with deficiencies in TLR2, TLR4 or TLR6. The possible implications for protection against and pathogenesis of bacterial infections are discussed.

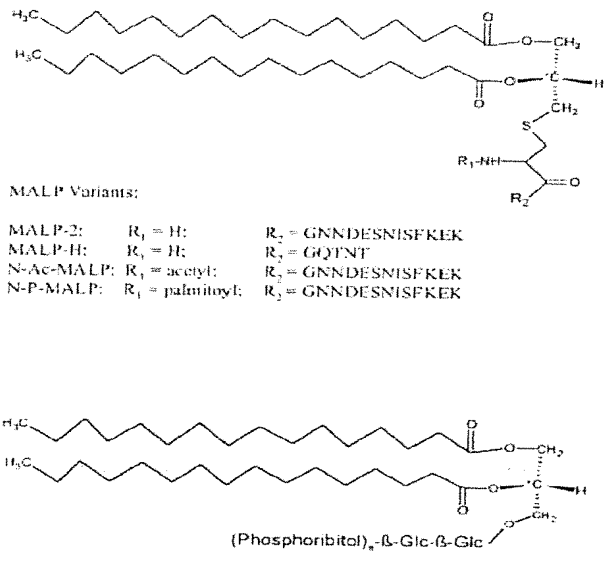

Fig. 1. Structures of synthetic MALP variants, and of lipoteichoic acid from *S. aureus*. Note the similarities of the lipid moieties when $R_1 = H$, which is the case in mycoplasma-derived MALP. In MALP-2 sulfoxide one oxygen is added to the sulfur of MALP-2. The lipid is drawn in the natural and active 2*R* configuration, and the asymmetric carbon atom 2 in the lipid moiety marked with *.

2 Results

2.1 Differential recognition of MALP-2 variants, *Borrelia* lipopeptide and LTA by TLR2 and TLR6

Earlier data from our laboratories had shown that MALP-2-dependent Mϕ activation requires recognition by functional TLR2 and TLR6 [20]. In order to further our understanding of the molecular basis for lipopeptide TLR interactions, synthetic variants of MALP-2, *i.e.* N-acetyl-MALP-2, N-palmitoyl-MALP-2 and MALP-H, a synthetic lipopeptide originally derived from *M. hyorhinis* with the same lipid moiety but a different and shorter peptide sequence [22], were tested for their potential to induce monocyte chemoattractant protein-1 (MCP-1) release in fibroblast lines from TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR6$^{-/-}$ mice. The appropriate wild-type (WT) fibroblasts served as positive controls. Note that with one exception (racemic MALP-H) the natural 2*R* stereoisomers (see Fig. 1) of MALP variants were used in these and following experiments. Fibroblast lines were either from embryos (TLR2$^{-/-}$, TLR6$^{-/-}$) or from lung tissue of adult mice (TLR4$^{-/-}$). WT fibroblasts reacted to MALP-2 by secretion of IL-6 and the chemokines macrophage inflammatory protein-2 and MCP-1 (data not shown). We chose to use MCP-1 as readout system because this chemokine was produced in concentrations which reached those produced by peritoneal exudate cells on a per cell basis.

As shown in Fig. 2 WT fibroblasts from either C3H/HeN mice or F_2 interbreds from 129/Ola×C57BL/6 released significant amounts of MCP-1 in response to MALP-2, MALP-H, N-acetyl-MALP-2 or N-palmitoyl-MALP-2. LPS and LTA served as positive controls in these and following experiments. The latter modulin carries only two ester-bound long-chain fatty acids like MALP-2 and MALP-H (Fig. 1). Concentrations of stimulators were used that gave plateau values (dose response curves not shown). As expected from previous studies [17], TLR2$^{-/-}$ fibroblasts did not react to any of the tested modulins except LPS (Fig. 3). Incidentally, this demonstrates that

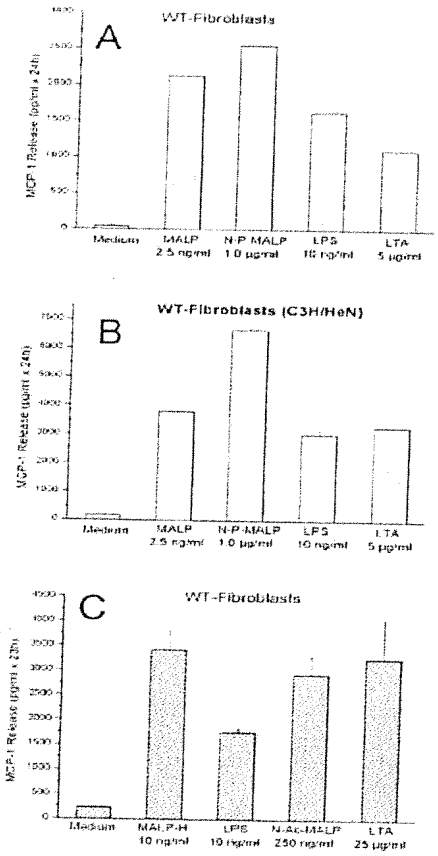

Fig. 2. MCP-1 release from WT fibroblasts in response to various modulins. WT fibroblasts were from embryos of 129/Ola×C57BL/6 mice (A, C), or lung fibroblasts from C3H/HeN mice (B), and were stimulated with the indicated concentrations of modulins for 24 h. MALP = *R*-MALP-2; N-P-MALP = N-palmitoyl-*S*-MALP-2; N-Ac-MALP = N-acetyl-*R*-MALP-2; MALP-H = *R*-[2,3-bis(palmitoyloxy)-(2*R*,*S*)-propyl]-cysteinyl-GQTNT, a synthetic analogue of a *M. hyorhinis*-derived lipopeptide; LPS = *S. typhimurium* lipopolysaccharide; LTA = *S. aureus* lipoteichoic acid. The chemokine was determined by ELISA. Data are means from duplicate cultures ± standard deviation.

the LTA preparation used was not contaminated by LPS. In contrast, TLR6$^{-/-}$ fibroblasts were only stimulated by N-palmitoyl-MALP-2 and LPS but neither by MALP-2 nor MALP-H (Fig. 4A, B). Surprisingly, TLR6$^{-/-}$ fibroblasts showed no response above the medium control to N-acetyl-MALP-2 (Fig. 4B). The response of TLR6$^{-/-}$ fibroblasts to LTA was poor (Fig. 4A, B) when compared to

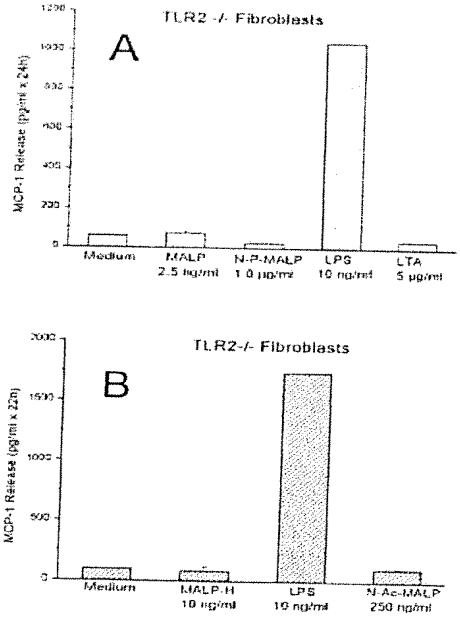

Fig. 3. MCP-1-release from embryonal TLR2$^{-/-}$ fibroblasts from 129/Ola×C57BL/6 mice in response to various modulins. Abbreviations as in Fig. 2. Data in A and B were from separate experiments. The cells were stimulated, and MCP-1 was determined as in Fig. 2. Data are means from duplicate cultures ± standard deviation.

the appropriate WT fibroblasts where LTA gave a response that was comparable to that of LPS, which served as positive control in these experiments (Fig. 2A, C). Since there was some controversy in the literature about the recognition of LTA by TLR [16, 21], this preparation of LTA was also tested in TLR4$^{-/-}$ fibroblast cultures and the appropriate control cells along with MALP, N-palmitoyl-MALP-2 and LPS as controls (see Fig. 4C). All preparations except LPS were able to stimulate chemokine release in this system. Finally, when recombinant preparations of tri-lipidated Lip-OspA or the truncated, non-lipidated and MDP-modified OspA peptide from *B. burgdorferi* were tested on fibroblasts from normal and TLR-deficient mice, MCP-1 release was only induced by Lip-OspA, but not MDP-OspA and only in WT and TLR6$^{-/-}$, but not in TLR2$^{-/-}$ cells (Fig. 5).

2.2 Variations of the lipid moiety of MALP-2 lead to altered specific activity

The determination of the stable decay products of nitric oxide, nitrite and nitrate in response to a serial dilution of

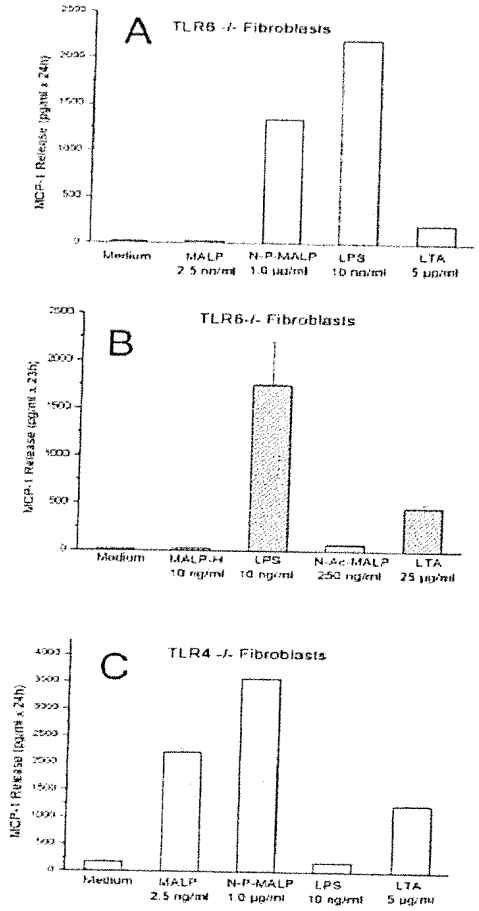

Fig. 4. MCP-1-release from TLR6$^{-/-}$ and TLR4$^{-/-}$ fibroblasts in response to various modulins. A, B: data are from two separate experiments wih embryonal fibroblasts from TLR6$^{-/-}$ 129/OlaxC57BL/6 mice, C: lung fibroblasts from C3H/HeJ mice. Abbreviations of modulins in legends to Fig. 2. The cells were stimulated, and MCP-1 was determined as in Fig. 2. Data are means from duplicate cultures ± standard deviation.

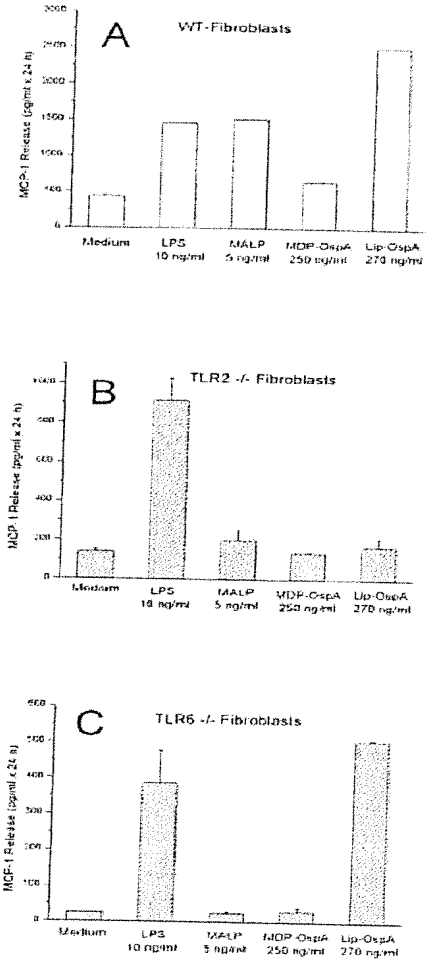

Fig. 5. MCP-1-release from WT versus TLR2$^{-/-}$ and TLR6$^{-/-}$ fibroblasts in response to *Borrelia burgdorferi* lipopeptide Lip-OspA and the respective peptide MDP-OspA in which the lipid moiety is replaced by the tripeptide MDP. The cells were stimulated, and MCP-1 was determined as in Fig. 2. Data are means from duplicate cultures ± standard deviation.

the stimulant in the presence of IFN-γ is generally used as a suitable, quantifiable *in vitro* assay of Mϕ activation [23]. For the following experiments we used primary resident peritoneal exudate Mϕ from LPS low responder mice as cell source. For a meaningful comparison of the stimulatory activities of various MALP-2 variants, the concentration at half-maximal activity (turning point of the dose response curve) was taken as criterion for specific activity, rather than the maximal attainable nitric oxide release, which may differ within separate experiments.

A comparison of stimulatory activities of lipopeptides from *M. hyorhinis* and *E. coli*, respectively, had suggested that N-acyl substitution with palmitic acid leads to lower specific activity [22]. Indeed, when MALP-2 was substituted with a third palmitic acid, specific activity

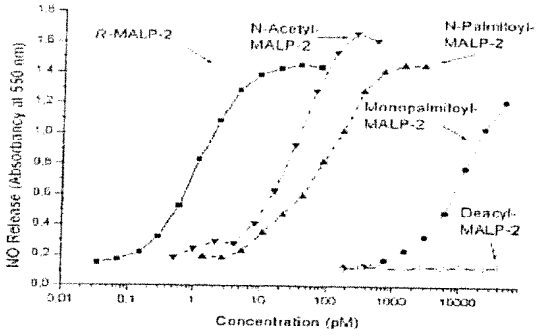

Fig. 6. Comparative dose response of variants of *R*-MALP-2. Nitric oxide release from C3H/HeJ mouse resident peritoneal exudate Mϕ was measured as the sum of nitrite and nitrate after 48 h stimulation with the indicated concentrations of the lipopeptides. Data were from triplicate cultures ± standard deviation.

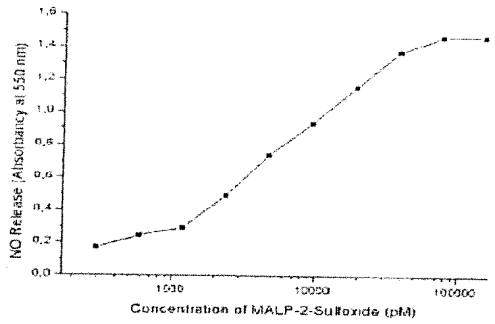

Fig. 7. Dose response curve of *R*-MALP-2 sulfoxide. Nitric oxide-release was measured as in Fig. 6. Note the different scale of the x axis in Fig. 6.

was reduced by a factor of about 50 (Fig. 6). In order to test whether this was due to bulkiness of this substituent or loss of the positive charge at the N terminus, the N terminus of cysteine was acetylated. Note that substitution with the comparatively small acetyl group led to a similar shift to lower activity as did substitution by palmitic acid (Fig. 6), although N-Acetyl-MALP-2 was recognized by the same combination of TLR2 and TLR6 as MALP-2. When one of the two ester-bound fatty acids was removed from MALP-2, the resulting activity of monopalmitoyl-MALP-2 was reduced by four orders of magnitude. MALP-2 without any fatty acids (deacyl-MALP-2, *i.e.* *R*-[(2,3-dihydroxy)-(2*R*)-propyl]-cysteinyl-GNNDESNISFKEK) showed no activity at all (Fig. 6). The data indicate that esterification of the lipid moiety of a bacterial lipoprotein by two fatty acids is sufficient to optimally stimulate the innate immune system via the concerted action of TLR2 and TLR6. This also explains the exceptionally high biological activity of mycoplasma-derived lipoproteins and -peptides on both Mϕ and fibroblasts as compared to lipoproteins from other bacteria, which mostly carry three long-chain fatty acids (see *e.g.* [22]).

2.3 Inactivation of MALP-2

As shown above, de-esterified MALP-2 loses much of its biological activity. A second inactivation mechanism which can be envisaged to occur *in vivo*, *e.g.* during an infection with mycoplasmas, is oxidation of the thioether group in MALP-2. Activated Mϕ and PMN are capable of generating active oxygen species [1]. The thioether group of MALP-2 is expected to be similarly reactive and prone to oxidation as that in methionine. Methionyl groups in proteins are known to be easily oxidized to the sulfoxide, be it spontaneously or enzymatically, and in particular in inflamed tissue (see review in [24]). It was of interest to see whether oxidation of the thioether group would result in an inactive MALP-2 species. To this end MALP-2 sulfoxide, which is the likely product of the first oxidation step, was synthesized and tested in the nitric oxide-release assay. As shown in Fig. 7, oxidation to the sulfoxide resulted in lowering the specific activity by four orders of magnitude when compared to MALP-2. We next studied the stability of MALP-2 in cell homogenates of peritoneal cells which were enriched with neutrophils by intraperitoneal injection of MALP-2 24 h prior to cell harvesting [25] thus mimicking the scenario at an inflammatory site rich in activated Mϕ and PMN. Cells were harvested, divided into two equal portions and sonicated. One portion was heat-inactivated, while the other was left at 0°C. MALP-2 was added to both samples and biological activity tested after various times at 37°C. As seen in Fig. 8, MALP-2 was time-dependently inactivated in both samples, however appreciably faster in the non-denatured homogenate, as opposed to the heat-inactivated one.

3 Discussion

3.1 Structural details which determine TLR usage

Our data show, in accordance with previously published data [20], that recognition of microbial lipopeptides carrying only two fatty acids requires co-expression of TLR2 and TLR6, while activation of cells via lipopeptide molecules containing three long-chain fatty acids like

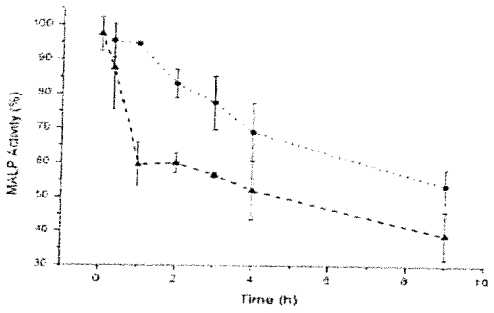

Fig. 8. Inactivation of MALP-2 in non-denatured and heat-treated homogenates of peritoneal cells enriched in neutrophils. NMRI mice were treated one day prior to cell harvesting with 2.5 μg *R*-MALP-2. A total of 10^6 peritoneal cells was harvested from ten mice and pooled. The cells, containing 28% PMN, were homogenized by sonication in two equal portions, one of which was heat-inactivated. *R*-MALP-2 was added to each sample and aliquots were taken after the indicated times and the remaining MALP-2 was assayed by the nitric oxide-release test as in Fig. 6. Triangles: non-denatured, circles: heat-inactivated homogenate. The data are means of two separate experiments with pooled cells from ten mice each ± standard deviation.

N-palmitoyl-MALP-2 is solely dependent on TLR2 (summarized in Table 1). The data further indicate that a short, N-linked acetyl group in addition to two long-chain fatty acids is insufficient to stimulate target cells via TLR2 alone. Based on these data we like to put forth the more general hypothesis that the TLR system can differentiate between bacterial modulins with two and those with three long-chain fatty acids in their lipid moiety, in that compounds with three fatty acids are recognized by TLR2, whereas signaling of those with two long-chain fatty acids requires the additional cooperation with TLR6. This notion does not exclude the cooperation of other yet undefined accessory molecules for signaling but they suggest the minimal TLR requirements. The hypothesis was tested by using LTA, a modulin that has a lipid moiety which is similar to that of bacterial lipopeptides and is also biochemically derived from phospholipid (see Fig. 1). However, it is linked to the polar moiety by a glycosidic, not a thioether bond [21]. The poor responsiveness of $TLR6^{-/-}$ fibroblasts to LTA supports our hypothesis, as the MCP-1 response in $TLR6^{-/-}$ versus WT fibroblasts was diminished by >80% at two different concentrations of LTA (Fig. 2A, B and 4A, C). The LTA used in this study showed no activity in the $TLR2^{-/-}$ fibroblasts (Fig. 3A), indicating that it was free of LPS. It did, however, stimulate $TLR4^{-/-}$ fibroblasts (Fig. 4C). This is in contrast to previous results [16] but in keeping with other publications [21]. We ascribe this difference to the different modes of purifying the LTA and to the fact that the formerly used LTA was from commercial sources. The partial decomposition and heterogeneity of commercial LTA preparations was the subject of a recent study [26].

The above hypothesis was further tested with recombinant preparations of Lip-OspA from *B. burgdorferi*. While the de-lipidated form, MDP-OspA (Fig. 5), as also

Table 1. Response patterns of MALP variants and LTA with respect to TLR usage and biological activity

Modulin	No. of long chain fatty acids	Free N-terminal amino group	TLR2 required	TLR6 required	MΦ stimulatory activity		
					high	medium	low
MALP-2	2	yes	yes	yes	+		
MALP-H	2	yes	yes	yes	+		
N-Ac-MALP-2	2	no	yes	yes		+	
Monoacyl-MALP-2	1	yes				inactive	
MALP-2 sulfoxide	2	yes				inactive	
N-P-MALP-2	3	no	yes	no		+	
Lip-OspA	3	no	yes	no		nt[a]	
LTA	2	no[b]	yes	yes			+

[a] nt = not tested in the nitric oxide-release assay.
[b] LTA contains no peptide in the lipid moiety.

deacyl-MALP-2 (Fig. 6), showed no activity at all, stimulation of fibroblasts by this lipoprotein was solely dependent of TLR2 and independent of TLR6. This result was not unexpected in light of the fact that this preparation of Lip-OspA, which is a component of a human vaccine against Lyme disease [27, 28], was expressed in *E. coli* and represents a tri-lipidated lipoprotein. However, our results contrast a recent study reporting that activation by Lip-OspA is critically dependent on the cooperation between TLR2 and TLR6 [18]. The origin of the OspA preparation, termed OspA-L, is not stated in this report, but may be a native form. This is of relevance in the context of a recent structural analysis of native lipoproteins of *B. burgdorferi* [29]. It was demonstrated that the respective lipopeptide contains two long-chain fatty acids, one in ester-linkage and one amide-bound, with one ester-bound acetyl group. It is possible that the fatty acid substitution of *Borrelia* lipoproteins is dependent on the environment and that those lipoproteins which express only two long fatty acids, such as the one described by Beerman et al. [29], require the co-expression of TLR2 and TLR6 on mammalian cells for an efficient stimulation of host cells. If in fact the native Lip-OspA were comparably active as other lipopeptides with two ester-linked fatty acids (see Fig. 6), a small content of a lipoprotein equipped with two ester-linked fatty acids would dominate the response of the tri-lipidated form and result in TLR6 usage (see Sect. 3.3).

3.2 Bacterial lipopeptides can stimulate fibroblasts to chemokine release

The analysis of TLR-mediated stimulation by MALP-2 and its variants as well as OspA and LTA was for technical reasons performed with TLR-deficient and WT fibroblasts as indicator cells. The fact that fibroblasts react to MALP-2 is in itself a new and interesting finding. MALP-2-stimulated fibroblasts liberated as much MCP-1 on a per cell basis as Mϕ. This observation is important in that it explains how bacteria, and mycoplasmas in particular, can attract leukocytes to sites of infection in the body which are poor in Mϕ but rich in fibroblasts, as *e.g.* wounded skin. MCP-1 and other chemokines would then lead to infiltration of phagocytes as a first line of defense. However, this topic requires further studies, as fibroblasts require much more MALP-2 for stimulation of chemokine synthesis than do Mϕ. It may well depend on the bacterial load at the site of infection, whether a few skin Mϕ suffice to trigger a response to mycoplasmal lipoproteins or whether enough mycoplasmas are present to be able to stimulate *e.g.* skin fibroblasts in the mucosa by shedded pieces of membrane containing lipoproteins.

3.3 Structural details which determine specific activity

According to our data several structural details influence specific activity which we define as the concentration required to reach half maximal activity in the nitric oxide-release assay: (i) bacterial lipopeptides contain an asymmetric carbon atom in the lipid moiety (see Fig. 1). The TLR system discriminates between the two possible stereoisomers. We had previously reported that only the natural stereoisomer shows good activity [17]. It is really the *R*-isomer which is the natural and active one (see Fig. 6). (ii) A comparison of the specific activities of variants of this *R*-isomer showed that substitution of the N terminus with either an acetyl or palmitoyl group shifts the specific activity to lower values (Fig. 6). (iii) Removal of one or both ester-bound fatty acids lowered the specific activity by five orders of magnitude or deleted biological activity altogether, respectively (Fig. 6, see also [30]). We do not know which fatty acid was removed by mild alkaline hydrolysis, but according to the rules of organic chemistry it is likely to be preferentially the one esterifying the primary OH-group. During HPLC purification and concentration of the fractions, both under acidic conditions, an acyl migration is expected to occur [31], leading to S-[3(palmitoyloxy)-(2*R*)-propyl]-CGNNDESNISFKEK. Neither alkaline hydrolysis nor acyl migration is expected to change the asymmetry at carbon 2 of the lipid moiety. (iv) Oxidation of the thioether lowered the specific activity by more than four orders of magnitude (Fig. 7). Although an exact comparison of specific activities of lipopeptides with LTA is not very meaningful, it is likely that the lack of the thioether group in LTA contributes to the comparably much lower biological activity.

3.4 Inactivation of lipopeptides

The above results suggest that there are at least two ways in which lipoproteins and -peptides can be physiologically inactivated: by de-esterification and by oxidation. Both mechanisms are likely to occur in inflamed tissue which are rich in phagocytes actively producing activated oxygen species and esterases. An additional experiment showed that MALP-2 was indeed inactivated in homogenates of Mϕ and PMN. Inactivation occurred more rapidly in non-denatured homogenate than in a heat-inactivated control sample (Fig. 8). It was not feasible to isolate and characterize the decay product(s) of MALP-2 for lack of sufficient material. However, the close chemical similarity of the thioether in MALP-2 with that in methionine makes it likely that oxidation of such thioether groups occurs very easily and even spontaneously [24].

3.5 Implications for host-bacteria interactions

Lipoproteins are the most ubiquitous bacterial modulins. Pioneer studies from the laboratory of G. Jung (see *e.g.* [30]) and further data from other laboratories [22, 32] had already suggested that the biological activity of bacterial lipoproteins and -peptides is greatly dependent on the lipid part. The importance of this study lies in the identification of those structural details in the lipid moiety which determine receptor usage and biological activity. Thus, seemingly small structural modifications may lead to recognition by different TLR, to sometimes great changes in biological activity, or both. The implications for host-bacteria interactions are manifold, because such variations occur naturally. Not only do different species of bacteria synthesize lipoproteins with different variants of their lipid (*e.g.* compare *E. coli* [10], *B. burgdorferi* [29], *M. fermentans* and *M. hyorhinis* [9, 22]), but it is suggested by the data presented here that a host reaction, such as infiltration and activation of leukocytes, leads to modifications resulting in inactivation. It is further conceivable that, as recently shown for the lipid A in LPS [33], also lipoprotein structures may vary with the environmental conditions which these bacteria encounter upon colonization. The extent of a host reaction will again depend on these variations because (i) the biological activity depends on it, and (ii) at a given site of colonization different host cells being equipped with different sets of TLR [34] may respond to a bacterial challenge, depending on the type of lipoprotein formed by these bacteria.

4 Materials and methods

4.1 Synthesis of MALP-2 and variants

MALP-2 and its N-palmitoyl and N-acetyl derivatives were prepared essentially as described [9, 35]. Briefly, the tert-butyl and tert-butoxycarbonyl protected peptide GNNDESNISFKEK was synthesized on a Tentagel-PHB resin by Rapp Polymers, Tübingen, Germany. The N-fluorenylmethoxycarbonyl-protected S-[2,3-bis(palmitoyloxy)-(2*S*)-propyl]-L-cysteine or S-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteine isomers, respectively, were synthesized as described by Metzger et al. [35]. N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteine was prepared as reported by Metzger et al. [36]. The N-fluorenylmethoxycarbonyl-protected S-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteine sulfoxide was prepared according to Ravikumar et al. by oxidation of the N-fluorenylmethoxycarbonyl-protected S-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteine with 30% H_2O_2 in hexafluoro-2-propanol for 3 h at 25°C [37]. The respective lipid moieties were coupled to the resin-bound 13-mer with diisopropylcarbodiimide/N-hydroxybenzotriazole as described [30]. The N-fluorenylmethoxycarbonyl group was removed from the resin-bound N-fluorenylmethoxycarbonyl-protected S-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-CGNNDESNISFKEK with piperidine/dimethylformide. N-acetyl-MALP-2 was prepared by N-acetylation of the N-deprotected resin-bound MALP-2 with acetic anhydride in pyridine/dimethylformamide for 2 h at 25°C. Tert. butoxycarbonyl protecting groups were removed and the lipopeptides simultaneously cleaved off the carrier by a 4-h treatment at 25°C with trifluoroacetic acid containing 3% (vol/vol) water and 2% (vol/vol) triisobutylsilane. Monopalmitoyl-*R*-MALP-2 was prepared from *R*-MALP-2 by controlled alkaline hydrolysis in 0.01 N NaOH in 33% 2-propanol/water at room temperature for 1 h. During purification of the product by HPLC under acidic conditions (see below) a migration of the remaining palmitic acid to position 3 is to be expected [31], while the stereochemistry should be unaffected. MALP-H, S-[2,3-bis(palmitoyloxy)-(2*R,S*)-propyl]-cysteinyl-GQTNT, a synthetic analogue of a *M. hyorhinis*-derived lipopeptide, was synthesized as described [22].

4.2 HPLC purification of MALP-2 and its variants

Crude MALP-2 and variants thereof were purified by reversed phase HPLC on Nucleosil columns (Macherey & Nagel, Düren, Germany) as described [9, 22]. Elution was performed at 40°C with a linear water/2-propanol gradient containing 0.1% trifluoro acetic acid with a pump rate of 0.5 ml/min for the analytical 250/4 columns or 3 ml/min for the 250/10 columns, respectively. Elution of lipopeptides was monitored by determination of free amino acids with fluorescamine; active material was assayed with the nitric oxide-release assay (see below). The respective percentage of organic phase with which MALP-2 and variants are eluted is shown in Table 2. The final products were characterized by matrix-assisted laser desorption/ionization mass spectroscopy as described in detail [22]. The exact lipopeptide content was determined by amino acid analysis. MALP-2 and variants were kept as stock solutions water/2-propanol 1/1 (vol/vol) at 4°C. Unless stated otherwise the active *S*-isomer was used throughout in this study. The structures of MALP variants and LTA are compared in Fig. 1.

4.3 Preparation of LPS, LTA and recombinant OspA

LPS was from the smooth form of *Salmonella typhimurium* and phenol/water-purified as described [38]. LTA was isolated by a new method from *Staphylococcus aureus* (DSM 20233) as described [21]. Briefly, this method involves extraction of the bacteria with n-butanol and hydrophobic interaction chromatography on octyl Sepharose. Recombinant preparations of Lip-OspA and of its de-lipidated variant MDP-OspA, in which the signal sequence (16 amino acids + cysteine) was replaced by the three amino acids, methionine, aspartate and proline were expressed in *E. coli* as described [39, 40]. Quantitative analysis showed that the recombinant Lip-OspA is a tri-lipidated lipoprotein (R. Wallich, unpublished).

Table 2. Molecular mass and reversed phase HPLC of MALP-2 and variants thereof

Compound	Mol. Mass	Reversed phase column	Elution with 2-propanol (%)[a]
MALP-2	2,134	SP 250/10 Nucleosil 300-7 C8	53
N-palmitoyl-MALP-2	2,373	ET 250/4 Nucleosil 300-7 C4	42
N-acetyl-MALP-2	2,176	ET 250/4 Nucleosil 120-7 C18	85
Monopalmitoyl-MALP-2	1,897	ET 250/4 Nucleosil 120-5 C8	53[b]
MALP-2 sulfoxide	2,150	ET 250/4 Nucleosil 120-7 C18	74

[a] Elution from reversed phase columns. Elution was performed at 40°C with a linear water/2-propanol gradient containing 0.1% trifluoroacetic acid.
[b] MALP-2 elutes from this column with 65% 2-propanol.

4.4 Mice

Female C3H/HeJ LPS low-responder and C3H/HeN mice were from Jackson Laboratory (Bar Harbor, ME) and used at the age between 3 and 10 months. TLR2$^{-/-}$, TLR6$^{-/-}$ and the corresponding WT mice were F_2 interbreds from the 129/Ola×C57BL/6 F_1 strain and generated by gene targeting as described previously [12, 16, 20].

4.5 Nitric oxide-release assay

The nitric oxide-release assay was performed as described [9, 23]. Briefly, resident peritoneal exudate cells from C3H/HeJ mice served as the Mϕ source. They were seeded in 96-well microtiter plates and simultaneously stimulated with rIFN-γ (a generous gift of Dr. Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria) and a serial dilution of Mϕ-activating material. MALP-2 and variants thereof were solubilized in 25 mM octyl glucoside in the first dilution step and then further diluted with medium. After an incubation period of 45–48 h, nitrate was reduced with nitrate reductase (Boehringer), and nitric oxide determined as the sum of nitrate plus nitrite, using Griess reagent.

4.6 Inactivation of MALP-2 in homogenates of peritoneal cells enriched in PMN

Ten NMRI mice were each given an intraperitoneal injection of 2.5 μg *R*-MALP-2. A total of 10^8 peritoneal cells was harvested one day later and pooled. The cells, containing 28% neutrophils, were suspended in 9 ml Dulbecco's phosphate buffered saline, pH 6.2, and divided into two equal portions. Both were homogenized by two 20 s bursts of sonication with a microtip (Branson Sonifier) while in an ice bath. One portion was treated for 3 min in a boiling water bath. *R*-MALP-2 (21 μg) in 0.8 ml 25 mM octyl glucoside was added to each sample and aliquots were taken after the indicated times and heated in 25 mM octyl glucoside to stop the reaction and extract the remaining MALP-2 which was assayed by the nitric oxide release test. Two separate experiments were carried out with peritoneal cells from ten mice each.

4.7 Fibroblasts

Embryonal fibroblasts were obtained by dissecting 14-day-old embryos from 129Ola×C57BL/6 mice into small pieces and digesting these in 0.25% trypsin-EDTA at room temperature. Digestion was stopped by adding DMEM with 10% FCS. Single cells were then suspended by pipetting and plated in DMEM with 10% FCS. Lung fibroblasts from grown-up C3H/HeJ (TLR4$^{-/-}$ [13]) or C3H/HeN (WT) mice were generated by covering small pieces of lung tissue in petri dishes with microscope slides and incubating them for 2–3 weeks in RPMI 1640 and DMEM 1+1 medium with 15% FCS- and 10% MALP-2-conditioned medium. MALP-2-conditioned medium was generated by incubating 2–3×10^7 murine peritoneal cells with 80 pg/ml MALP-2 for 20 h. After this time the medium was removed, cell debris centrifuged off and the supernatant solution passed through a 0.2-μm filter.

4.8 MCP-1-release assay

Trypsinized fibroblasts were seeded at 7×10^3 cells/well in 200 μl volumes of DMEM-RPMI 1640 medium (1+1), 10% FCS, into 96-well flat-bottom microtiter plates. After overnight cultivation the medium was replaced by fresh medium with the indicated concentrations of stimulants. The culture medium was harvested after 24 h and assayed for MCP-1 with a commercial ELISA kit from R&D Systems (Wiesbaden-Nordernstadt, Germany). The assay was performed according to the manufacturer's protocol.

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